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TITLE OF INVENTION: FARNESYL DIBENZODIAZEPINONE, PROCESSES FOR ITS PRODUCTION AND ITS USE AS A PHARMACEUTICAL

RELATED APPLICATIONS

This application claims priority to U.S. Provisional Application 60/441,126, filed January 21, 2003; U.S. Provisional Application 60/492,997, filed August 7, 2003; and U.S. Provisional Application 60/518,286, filed November 10, 2003. The entire teachings of the above provisional applications are incorporated herein by reference.

FIELD OF THE INVENTION

This invention relates to a novel farnesylated dibenzodiazepinone, named ECO-04601, its pharmaceutically acceptable salts and derivatives, and to methods for obtaining the compound. One method of obtaining the compound is by cultivation of a novel strain of *Micromonospora sp.*, i.e., 046-ECO11 or [S01]046; another method involves expression of biosynthetic pathway genes in transformed host cells. The present invention further relates to *Micromonospora sp.* strains 046-ECO11 and [S01]046, to the use of ECO-04601 and its pharmaceutically acceptable salts and derivatives as pharmaceuticals, in particular to their use as inhibitors of cancer cell growth, bacterial cell growth, mammalian lipoxygenase, and for treating acute and chronic inflammation, and to pharmaceutical compositions comprising ECO-04601 or a pharmaceutically acceptable salt or derivative thereof. Finally, the invention relates to novel polynucleotide sequences and their encoded proteins, which are involved in the biosynthesis of ECO-04601.

BACKGROUND OF THE INVENTION

The euactinomycetes are a subset of a large and complex group of Gram-positive bacteria known as actinomycetes. Over the past few decades these organisms, which are abundant in soil, have generated significant commercial and scientific interest as a result of the large number of therapeutically useful compounds, particularly antibiotics, produced as secondary metabolites. The intensive search for

strains able to produce new antibiotics has led to the identification of hundreds of new species.

Many of the euactinomycetes, particularly Streptomyces and the closely related Saccharopolyspora genera, have been extensively studied. Both of these genera produce a notable diversity of biologically active metabolites. Because of the commercial significance of these compounds, much is known about the genetics and physiology of these organisms.

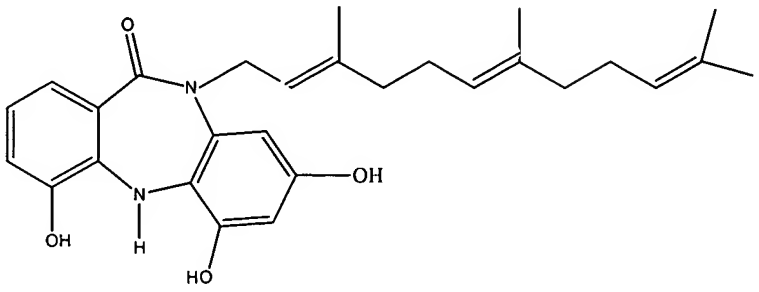
Another representative genus of euactinomycetes, Micromonospora, has also generated commercial interest. For example, U.S. Patent No. 5,541,181 (Ohkuma et al.) discloses a dibenzodiazepinone compound, specifically 5-farnesyl-4,7,9-trihydroxy-dibenzodiazepin-11-one (named "BU-4664L"), produced by a known euactinomycetes strain, *Micromonospora* sp. M990-6 (ATCC 55378). The Ohkurma et al. patent reports that BU-4664L and its chemically synthesized di- and tri-alkoxy and acyloxy derivatives possess anti-inflammatory and anti-tumor cell activities.

Although many biologically active compounds have been identified from bacteria, there remains the need to obtain novel naturally occurring compounds with enhanced properties. Current methods of obtaining such compounds include screening of natural isolates and chemical modification of existing compounds, both of which are costly and time consuming. Current screening methods are based on general biological properties of the compound, which require prior knowledge of the structure of the molecules. Methods for chemically modifying known active compounds exist, but still suffer from practical limitations as to the type of compounds obtainable.

Thus, there exists a considerable need to obtain pharmaceutically active compounds in a cost-effective manner and with high yield. The present invention solves these problems by providing a novel strain of Micromonospora capable of producing a potent new therapeutic compound, as well as reagents (e.g., polynucleotides, vectors comprising the polynucleotides and host cells comprising the vectors) and methods to generate novel compounds by de novo biosynthesis rather than by chemical synthesis.

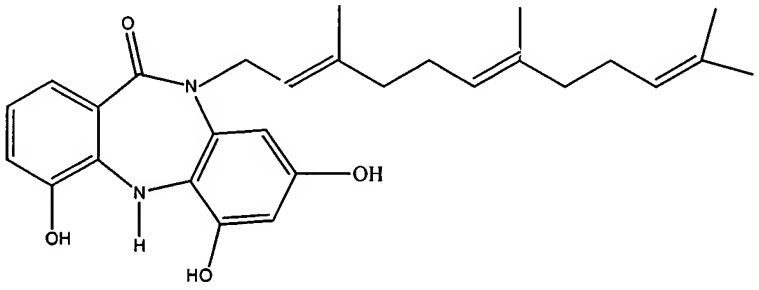
SUMMARY OF THE INVENTION

In one aspect, the invention relates to a compound of the formula



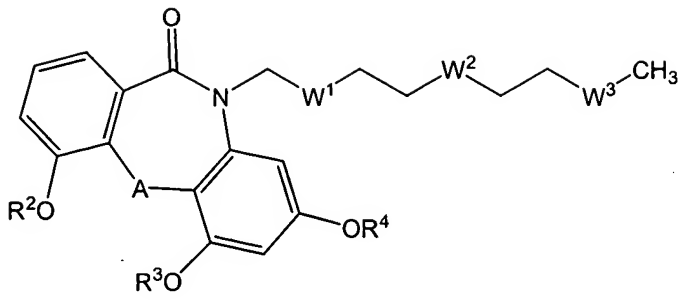
(Formula II) or a pharmaceutically acceptable salt thereof.

In another aspect, the invention relates to a pharmaceutical composition comprising a compound of the formula



or a pharmaceutically acceptable salt thereof, together with a pharmaceutically acceptable carrier.

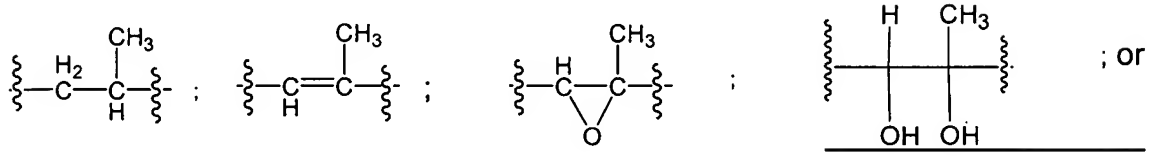
In a further aspect, the invention relates to a class of compounds represented by Formula I:



Formula I

wherein,

W1, W2 and W3 is each independently selected from



the chain from the tricycle may terminate at W3, W2 or W1 with W3, W2 or W1 respectively being either –CH=O or –CH2OH;

A is selected from –NH--, –NCH2R1, -NC(O)R1;

R1 is selected from C1-6 alkyl, C2-6 alkene, aryl or heteroaryl;

R2, R3, and R4 is each independently selected from H, R5, -C(O)R6

R5 is each independently selected from C_{1-6} alkyl, C_{2-7} alkalene, aryl or heteroaryl;

R6 is each independently selected from H, C_{1-6} alkyl, C_{2-7} alkalene, aryl or heteroaryl; or a pharmaceutically acceptable salt thereof.

In one embodiment, A is NH.

In another embodiment, A is $–NCH_2R^1$.

In another embodiment, A is $-NC(O)R^1$.

In another embodiment, R^2 is H.

In another embodiment, R^3 is H.

In another embodiment, R^4 is H.

In another embodiment, R^2, R^3 and R^4 are each H.

In another embodiment, R^2, R^3 and R^4 are each H, and W^1 is –CH =CH-.

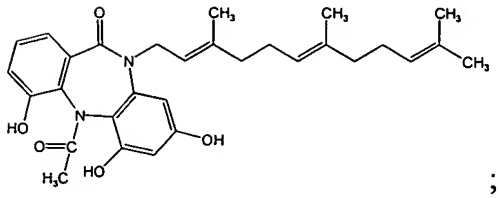
In another embodiment, R^2, R^3 and R^4 are each H, and W^2 is –CH =CH-.

In another embodiment, R^2, R^3 and R^4 are each H, and W^3 is –CH =CH-.

In another embodiment, A is NH and R^2, R^3 and R^4 are each H.

In another embodiment, A is NH, each of W^1, W^2, and W^3 is –CH =CH-.

The invention further encompasses a compound selected from the group consisting of:



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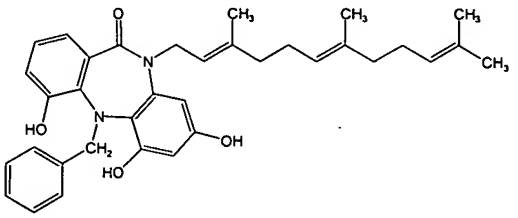

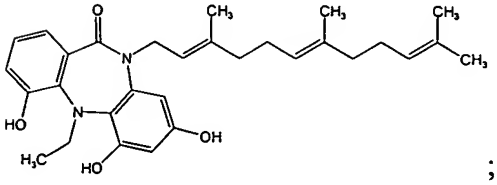

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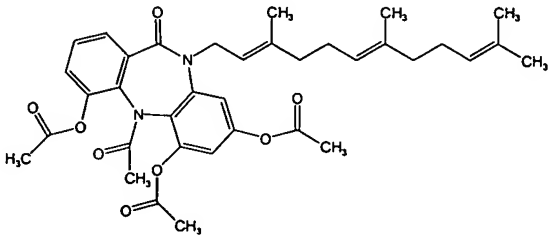

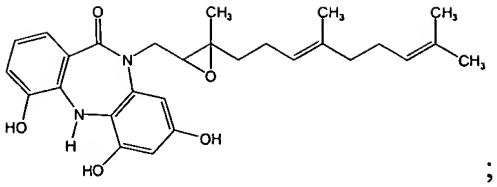
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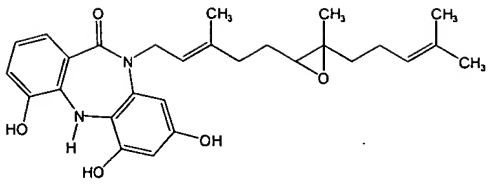

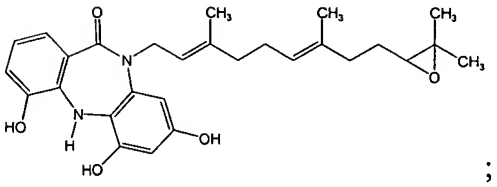
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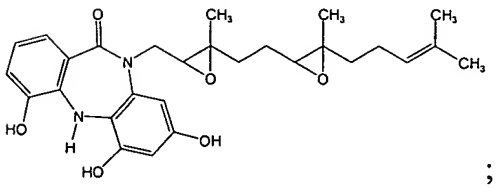
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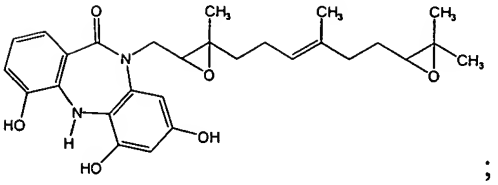
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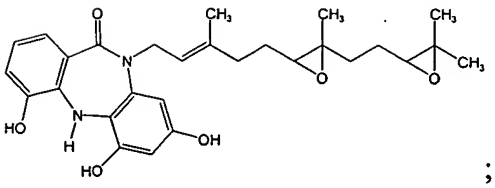
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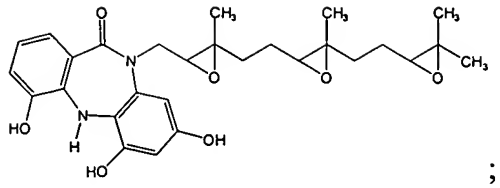
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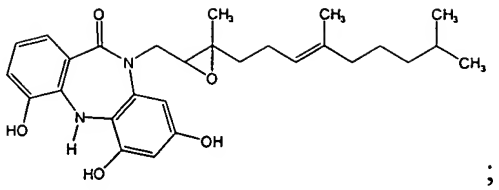
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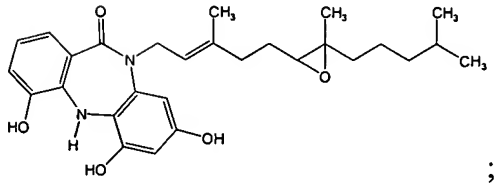
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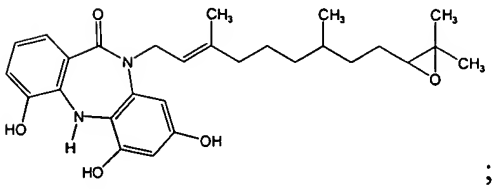
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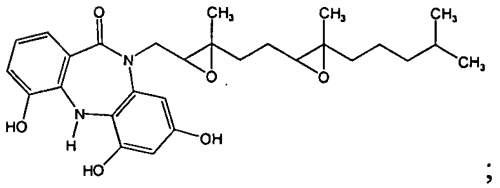
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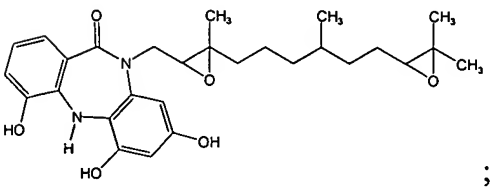
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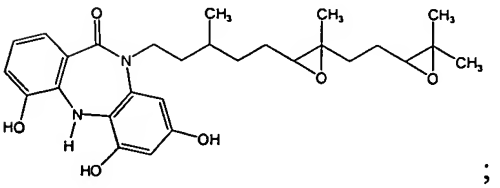
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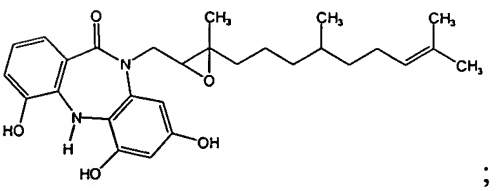
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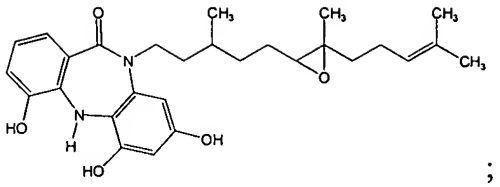
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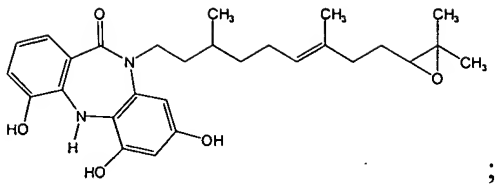
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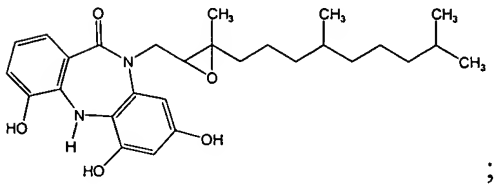
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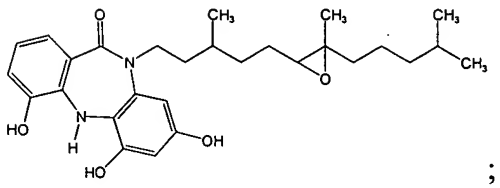
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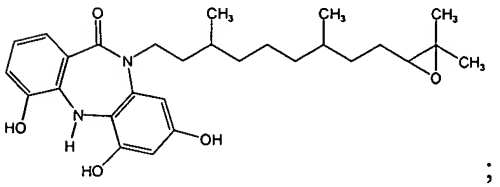
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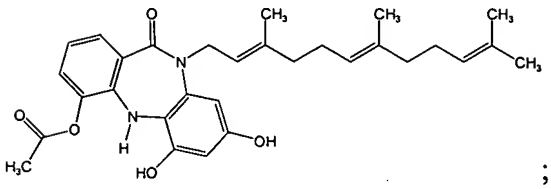
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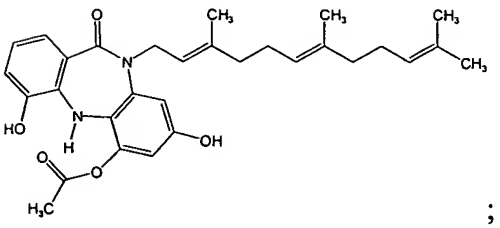
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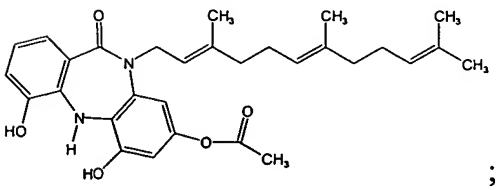
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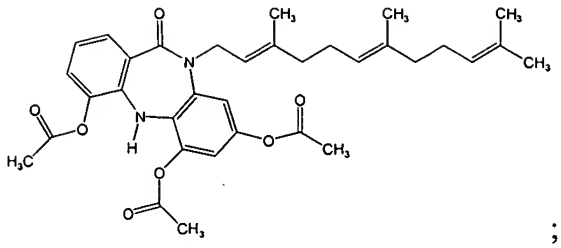
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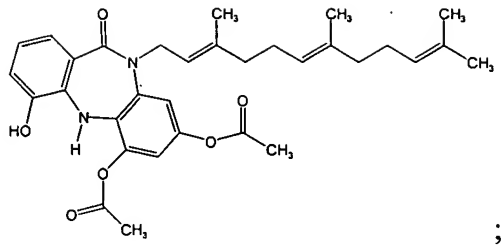
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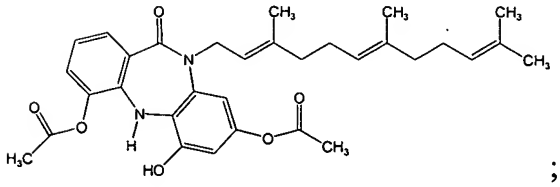
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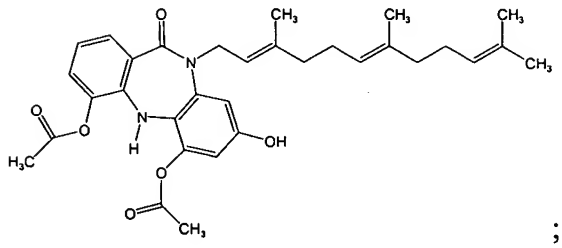
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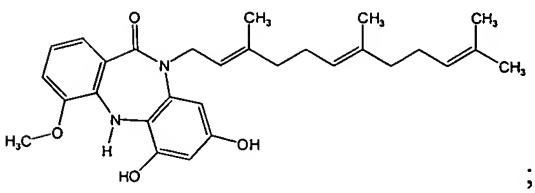
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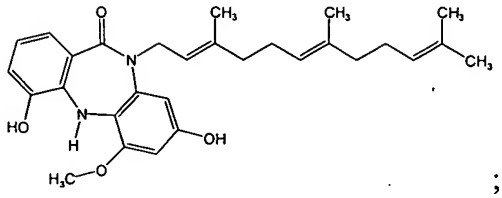
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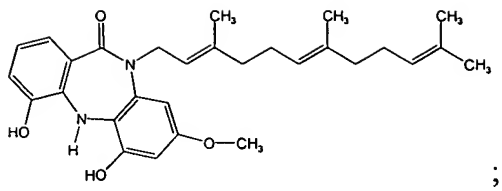
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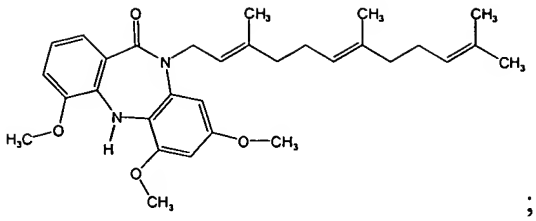
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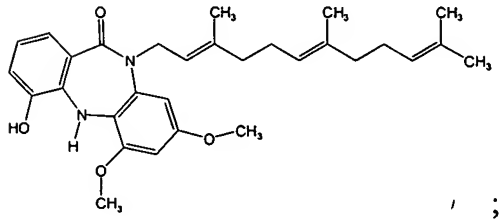
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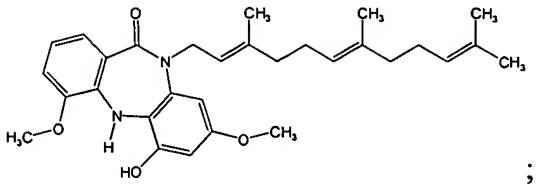
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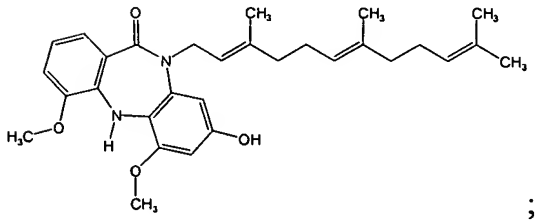
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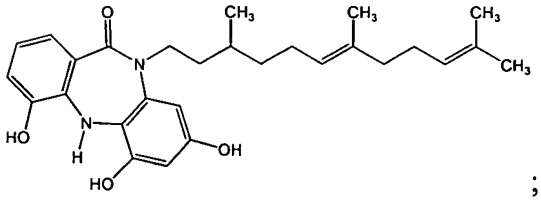
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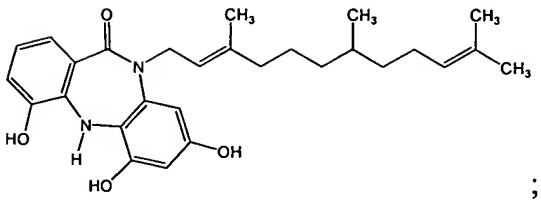
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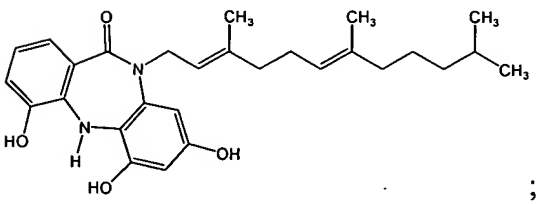
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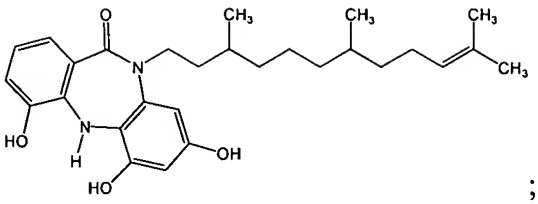
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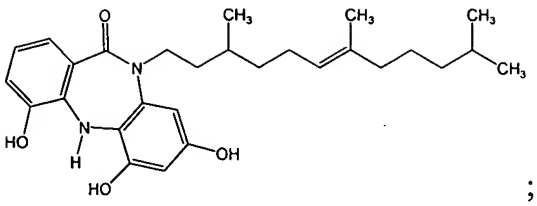
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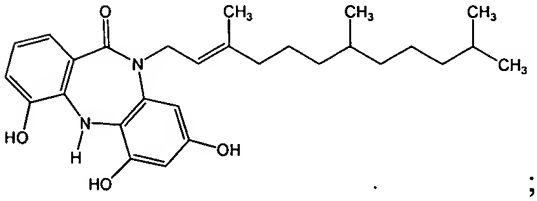
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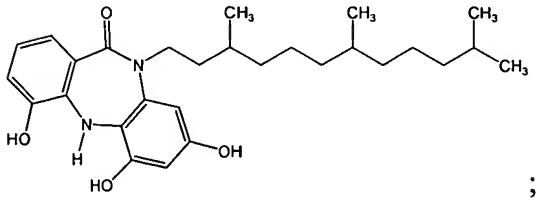
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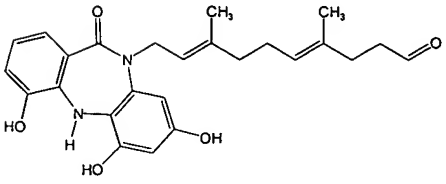
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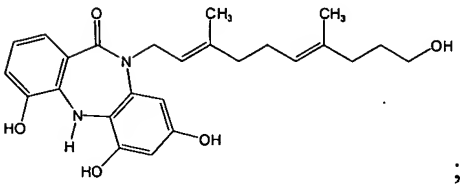
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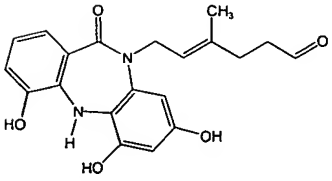
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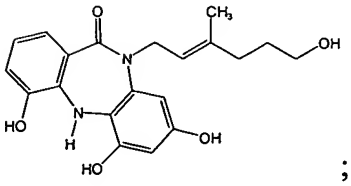
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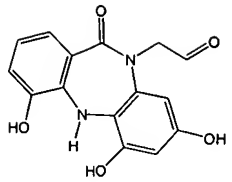
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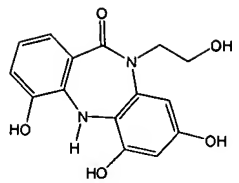
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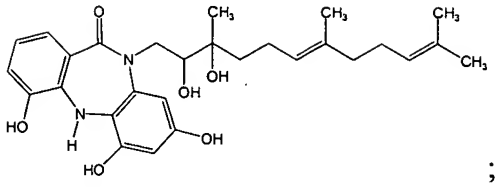
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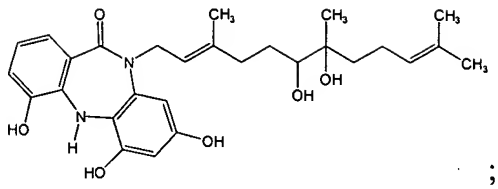
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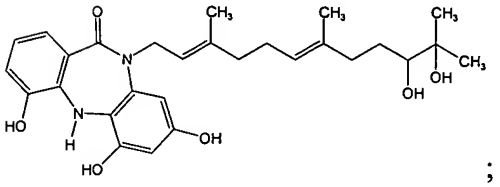
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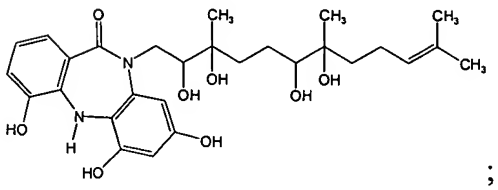
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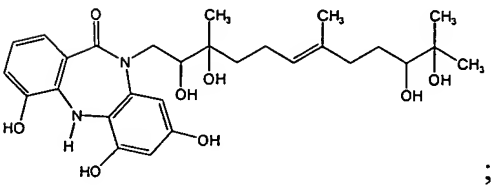
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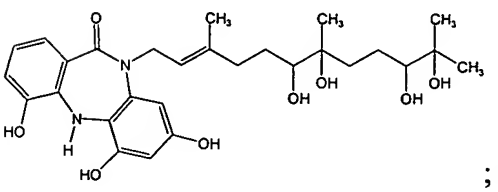
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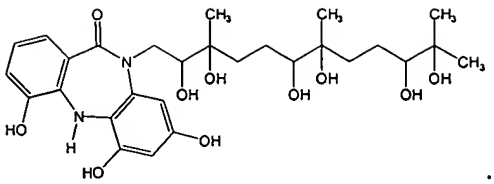


In one embodiment, the invention relates to compositions of the compounds of Formula I together with a pharmaceutically acceptable carrier.

The invention further encompasses a farnesyl dibenzodiazepinone obtained by a method comprising: a) cultivating *Micromonospora sp.* strain [S01]046, wherein the cultivation is performed under aerobic conditions in a nutrient medium comprising at least one source of carbon atoms and at least one source of nitrogen atoms; and b) isolating a farnesyl dibenzodiazepinone from the bacteria cultivated in step (a). In one embodiment the farnesyl dibenzodiazapinone is the compound of Formula II.

In one embodiment, the farnesyl dibenzodiazepinone generates NMR spectra essentially as shown in Figure 3, 4, 5, 6 and 7. In another embodiment, the farnesyl dibenzodiazepinone generates an ^{1}H NMR spectrum of Figure 3.

The invention further encompasses a process for making a farnesyl dibenzodiazapinone compound, comprising cultivation of *Micromonospora sp.* strain 046-ECO11, in a nutrient medium comprising at least one source of carbon atoms and at least one source of nitrogen atoms, and isolation and purification of the compound.

The invention further encompasses a process for making a farnesyl dibenzodiazepinone compound comprising cultivation of *Micromonospora sp.* strain [S01]046 in a nutrient medium comprising at least one source of carbon atoms and at least one source of nitrogen atoms, and isolation and purification of the compound.

In one embodiment, the cultivation occurs under aerobic conditions.

In another embodiment, the carbon atom and nitrogen atom sources are chosen from the components shown in Table 16.

In another embodiment, the cultivation is carried out at a temperature ranging from 18°C to 40°C. In a further embodiment, the temperature range is 18°C to 29°C.

In another embodiment, the cultivation is carried out at a pH ranging from 6 to 9.

The invention further encompasses the *Micromonospora sp.* having IDAC Accession No. 231203-01.

The invention further encompasses a method of inhibiting the growth of a cancer cell, the method comprising contacting the cancer cell with a compound of Formula I, such that growth of the cancer cell is inhibited.

In one embodiment, the compound is ECO-04601.

The invention further encompasses a method of inhibiting the growth of a cancer cell in a mammal, the method comprising administering a compound of Formula I to a mammal comprising a cancer cell, such that growth of the cancer cell is inhibited in the mammal.

In one embodiment, the compound is ECO-04601.

The invention further encompasses a method of treating a pre-cancerous or cancerous condition in a mammal, comprising the step of administering to the mammal a therapeutically effective amount of a compound of Formula I, such that a pre-cancerous or cancerous condition is treated.

In one embodiment, the compound is ECO-04601.

The invention further encompasses a method of treating a bacterial infection in a mammal, comprising administering a therapeutically effective amount of a compound of Formula I to a mammal having a bacterial infection, such that the bacterial infection is treated.

In one embodiment, the compound is ECO-04601.

The invention further encompasses a method of reducing inflammation in a mammal, comprising administering to a mammal having inflammation a therapeutically effective amount of a compound of Formula I, such that the inflammation is reduced.

In one embodiment, the compound is ECO-04601.

The invention further encompasses an isolated polynucleotide comprising one or more of SEQ ID NOs. 1, 64 and 73, wherein the polynucleotide encodes a polypeptide that participates in a biosynthetic pathway for a farnesyl dibenzodiazepinone.

The invention further encompasses an isolated polynucleotide comprising SEQ ID NOs. 1, 64 and 73, wherein the polynucleotide encodes a polypeptide that participates in a biosynthetic pathway for a farnesyl dibenzodiazepinone.

The invention further encompasses an isolated polynucleotide that encodes a polypeptide selected from the group consisting of SEQ ID NOs. 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 66, 68, 70, 72, 75, 77, 79, 81, 83, 85, 87 and 89.

In one embodiment, the isolated polynucleotide comprising SEQ ID No. 1 encodes a polypeptide selected from the group consisting of SEQ ID Nos. 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61 and 63.

In another embodiment, the isolated polynucleotide comprising SEQ ID No. 64 encodes a polypeptide selected from the group consisting of SEQ ID NOS: 66, 68, 70 and 72.

In another embodiment, the isolated polynucleotide comprising SEQ ID No. 73, encodes a polypeptide selected from the group consisting of SEQ ID NOS: 75, 77, 79, 81, 83, 85, 87 and 89.

The invention further encompasses an isolated polypeptide of SEQ ID NO. 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 66, 68, 70, 72, 75, 77, 79, 81, 83, 85, 87 or 89.

In one embodiment, the polypeptide participates in a biosynthetic pathway for a farnesyl dibenzodiazepinone.

The invention further encompasses an expression vector comprising one or more of the polynucleotides described herein.

The invention further encompasses a recombinant prokaryotic organism comprising one or more such expression vectors.

In one embodiment, the organism is an actinomycete.

In another embodiment, the organism requires the expression vector to synthesize a farnesyl dibenzodiazepinone. That is, the organism is deficient in the ability to synthesize a farnesyl dibenzodiazepinone before transformation with a polynucleotide as described herein.

The invention further encompasses a method of making a farnesyl dibenzodiazepinone *de novo* in a prokaryote, comprising the steps of: (a) providing a prokaryote that is incapable of synthesizing a farnesyl dibenzodiazepinone; (b) transforming the prokaryote with an expression vector as described herein; and (c) culturing the prokaryote; wherein the culturing results in the synthesis of a farnesyl dibenzodiazepinone in the prokaryote.

In one embodiment, the prokaryote is an actinomycete.

In another embodiment, the vector expresses a polypeptide of SEQ ID NO: 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 66, 68, 70, 72, 75, 77, 79, 81, 83, 85, 87 or 89.

Brief Description of the Figures

FIGURE 1 shows the mass of ECO-04601 determined by electrospray mass spectrometry to be 462.6.

FIGURE 2 shows the absorption spectrum of purified ECO-04601 with a UVmax at 230 nm and a shoulder at 290nm.

FIGURE 3 shows proton NMR data for the compound dissolved in MeOH-d_4.

FIGURE 4 shows multidimensional pulse sequences gDQCOSY.

FIGURE 5 shows multidimensional pulse sequences gHSQC.

FIGURE 6 shows multidimensional pulse sequences gHMBC.

FIGURE 7 shows multidimensional pulse sequences NOESY.

FIGURE 8 shows the *in vitro* anti-inflammatory activity of ECO-04601. Graph shows percent inhibition of 5-lipoxygenase activity plotted against the Log μM concentration of ECO-04601 and NDGA. Graph shows the EC_{50} of ECO-04601 to be 0.93μM.

FIGURE 9 shows inhibition of tumor growth resulting from administration of 10 to 30 mg/kg of ECO-04601 to glioblastoma-bearing mice beginning one day after tumor cell inoculation.

FIGURE 10 shows inhibition of tumor growth resulting from administration of 20-30 mg/kg of ECO-04601 to glioblastoma-bearing mice beginning ten days after tumor cell inoculation.

FIGURE 11 shows micrographs of tumor sections from mice bearing glioblastoma tumors and treated with saline or ECO-04601. The cell density of tumor treated with ECO-04601 appears decreased and nuclei from ECO-04601-treated tumor cells are larger and pynotic suggesting a cytotoxic effect.

FIGURE 12 shows the biosynthetic locus of ECO-04601, isolated from *Micromonospora sp.* strain 046-ECO11, including the positions of cosmids 046KM and 046KQ.

FIGURE 13 shows a schematic diagram of the biosynthetic pathway for the production of the farnesyl-diphosphate group of ECO-04601 with biosynthetic enzymes indicated by their ORF number and family designation.

FIGURE 14 shows a schematic diagram of the biosynthetic pathway for the production of (a) 3-hydroxy-anthranilate-adenylate, and (b) 2-amino-6-hydroxy-[1,4]benzoquinone components as specified by ORFs present in the locus encoding ECO-04601. Biosynthetic enzymes are indicated by their ORF number and family designation.

FIGURE 15 shows a schematic diagram of the biosynthetic pathway for the assembly of the ECO-04601 precursors, farnesyl-diphosphate, 3-hydroxy-anthranilate-adenylate and 2-amino-6-hydroxy-[1,4]benzoquinone.

Biosynthetic enzymes are indicated by their ORF number and family designation.

FIGURE 16 shows a sequence listing table indicating the SEQ ID NO. and function for each of the open reading frames (ORFs) of the 046D biosynthetic locus and the corresponding gene product.

FIGURE 17 shows results of the fatty acid analysis of *Micromonospora sp.* strain 046ECO11 (Accession No. IDAC 070303-01). Analysis was conducted using gas chromatography on fatty acid methyl esters (FAME).

FIGURE 18 illustrates the 16S ribosomal RNA analysis of *Micromonospora sp.* strain 046ECO11 (Accession No. IDAC 070303-01). Alignment of 16S ribosomal RNA sequences demonstrates the phylogenetic relatedness of *Micromonospora sp.* strain 046ECO11 (indicated as MID352 ECOPIA#1 con) to *Micromonospora chalcea.*

FIGURE 19 shows the complete ^{1}H and ^{13}C NMR assignments for ECO-04601 when measured in MeOH-d4.

FIGURE 20 provides a listing of nucleic acid and polypeptide sequences according to the invention.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a novel farnesyl dibenzodiazepinone, referred to herein as "ECO-04601," which was isolated from novel strains of actinomycetes, *Micromonospora sp.* strain 046-ECO11 and strain [S01]046. These microorganisms were analysed using gas chromatography as Fatty acid methyl esters (FAME) (*Figure 17*) 6S ribosomal RNA determination (*Figure 18*) and were found to belong to the genus of Micromonospora. These organisms were deposited on March 7, 2003, and December 23, 2003, respectively, with the International Depository Authority of Canada (IDAC), Bureau of Microbiology, Health Canada, 1015 Arlington Street, Winnipeg, Manitoba, Canada R3E 3R2, under Accession Nos. IDAC 070303-01 and IDAC

231203-01, respectively.

The invention further relates to pharmaceutically acceptable salts and derivatives of ECO-04601, and to methods for obtaining such compounds. One method of obtaining the compound is by cultivating *Micromonospora sp.* strain 046-ECO11, or a mutant or a variant thereof, under suitable Micromonospora culture conditions, preferably using the fermentation protocol described hereinbelow.

The invention also relates to a method for producing novel polyketide compounds, namely farnesyl dibenzodiazepinones, by selectively altering the genetic information of an organism. The present invention further provides isolated and purified polynucleotides that encode farnesyl dibenzodiazepinone domains, i.e., polypeptides from farnesyl dibenzodiazepinone-producing microorganisms, fragments thereof, vectors containing those polynucleotides, and host cells transformed with those vectors. These polynucleotides, fragments thereof, and vectors comprising the polynucleotides can be used as reagents in the above described method. Portions of the polynucleotide sequences disclosed herein are also useful as primers for the amplification of DNA or as probes to identify related domains from other farnesyl dibenzodiazepinone producing microorganisms.

The present invention also relates to pharmaceutical compositions comprising ECO-04601 and its pharmaceutically acceptable salts and derivatives. ECO-04601 is useful as a pharmaceutical, in particular for use as an inhibitor of cancer cell growth, bacterial cell growth, and mammalian lipoxygenase. The invention also relates to novel polynucleotide sequences and their encoded proteins, which are involved in the biosynthesis of ECO-04601.

The following detailed description discloses how to make and use ECO-04601 and compositions containing this compound to inhibit microbial growth and/or specific disease pathways.

Accordingly, certain aspects of the present invention relate to pharmaceutical compositions comprising the farnesylated dibenzodiazepinone compounds of the present invention together with a pharmaceutically acceptable carrier, methods of using the compositions to inhibit bacterial growth, and methods of using the pharmaceutical

compositions to treat diseases, including cancer, and chronic and acute inflammation.

I. Definitions

For convenience, the meaning of certain terms and phrases used in the specification, examples, and appended claims, are provided below.

As used herein, the term "farnesyl dibenzodiazepinone" refers to a class of dibenzodiazepinone compounds containing a farnesyl moiety. The term includes, but is not limited to, the exemplified compound of the present invention, 10-farnesyl-4,6,8-trihydroxy-dibenzodiazepin-11-one, which is referred to herein as "ECO-04601." As used herein, the term "farnesyl dibenzodiazepinone" includes compounds of this class that can be used as intermediates in chemical syntheses. As used herein, the term "alkyl" refers to linear or branched hydrocarbon groups. Examples of alkyl groups include, without limitation, methyl, ethyl, n-propyl, isopropyl, n-butyl, pentyl, hexyl, heptyl, cyclopentyl, cyclohexyl, cyclohexymethyl, and the like. Alkyl may optionally be substituted with substituents selected from acyl, amino, acylamino, acyloxy, carboalkoxy, carboxy, carboxyamido, cyano, halo, hydroxyl, nitro, thio, alkyl, alkenyl, alkynyl, cycloalkyl, heterocyclyl, aryl, heteroaryl, alkoxy, aryloxy, sulfinyl, sulfonyl, oxo, guanidino and formyl.

The term "alkenyl" refers to linear, branched or cyclic hydrocarbon groups containing at least one carbon-carbon double bond. Examples of alkenyl groups include, without limitation, vinyl, 1-propen-2-yl, 1-buten-4-yl, 2-buten-4-yl, 1-penten-5-yl and the like. Alkenyl may optionally be substituted with substituents selected from acyl, amino, acylamino, acyloxy, carboalkoxy, carboxy, carboxyamido, cyano, halo, hydroxyl, nitro, thio, alkyl, alkenyl, alkynyl, cycloalkyl, heterocyclyl, aryl, heteroaryl, alkoxy, aryloxy, sulfinyl, sulfonyl, formyl, oxo and guanidino. The double bond portion(s) of the unsaturated hydrocarbon chain may be either in the cis or trans configuration.

The terms "cycloalkyl" and "cycloalkyl ring" refer to a saturated or partially unsaturated carbocyclic ring in a single or fused carbocyclic ring system having from three to fifteen ring members. Examples of cycloalkyl groups include, without limitation, cyclopropyl, cyclobutyl, cyclohexyl, and cycloheptyl. Cycloalkyl may optionally be

substituted with substituents selected from acyl, amino, acylamino, acyloxy, carboalkoxy, carboxy, carboxyamido, cyano, halo, hydroxyl, nitro, thio, alkyl, alkenyl, alkynyl, cycloalkyl, heterocyclyl, aryl, heteroaryl, alkoxy, aryloxy, sulfinyl, sulfonyl and formyl.

The terms "heterocyclyl" and "heterocyclic" refer to a saturated or partially unsaturated ring containing one to four hetero atoms or hetero groups selected from O, N, NH, NRx, PO2, S, SO or SO2 in a single or fused heterocyclic ring system having from three to fifteen ring members. Examples of a heterocyclyl or heterocyclic ring include, without limitation, morpholinyl, piperidinyl, and pyrrolidinyl. Heterocyclyl, heterocyclic or heterocyclyl ring may optionally be substituted with substituents selected from acyl, amino, acylamino, acyloxy, oxo, thiocarbonyl, imino, carboalkoxy, carboxy, carboxyamido, cyano, halo, hydroxyl, nitro, thio, alkyl, alkenyl, alkynyl, cycloalkyl, heterocyclyl, aryl, heteroaryl, alkoxy, aryloxy, sulfinyl, sulfonyl and formyl.

The term "amino acid" refers to any natural amino acid, all natural amino acids are well known to a person skilled in the art.

The term "halo" refers to a halogen atom, e.g., bromine, chlorine, fluorine and iodine.

The terms "aryl" and "aryl ring" refer to aromatic groups in a single or fused ring system, having from five to fifteen ring members. Examples of aryl include, without limitation, phenyl, naphthyl, biphenyl, terphenyl. Aryl may optionally be substituted with one or more substituent group selected from acyl, amino, acylamino, acyloxy, azido, alkythio, carboalkoxy, carboxy, carboxyamido, cyano, halo, hydroxyl, nitro, thio, alkyl, alkenyl, alkynyl, cycloalkyl, heterocyclyl, aryl, heteroaryl, alkoxy, aryloxy, sulfinyl, sulfonyl and formyl.

The terms "heteroaryl" and "heteroaryl ring" refer to aromatic groups in a single or fused ring system, having from five to fifteen ring members and containing at least one hetero atom such as O, N, S, SO and SO2. Examples of heteroaryl groups include, without limitation, pyridinyl, thiazolyl, thiadiazoyl, isoquinolinyl, pyrazolyl, oxazolyl, oxadiazoyl, triazolyl, and pyrrolyl groups. Heteroaryl groups may opitionally be substituted with one or more substituent group selected from acyl, amino, acylamino,

acyloxy, carboalkoxy, carboxy, carboxyamido, cyano, halo, hydroxyl, nitro, thio, thiocarbonyl, alkyl, alkenyl, alkynyl, cycloalkyl, heterocyclyl, aryl, heteroaryl, alkoxy, aryloxy, sulfinyl, sulfonyl, and formyl.

The terms "aralkyl" and "heteroaralkyl" refer to an aryl group or a heteroaryl group, respectively bonded directly through an alkyl group, such as benzyl. Aralkyl and heteroaralkyl may be optionally substituted as the aryl and heteroaryl groups.

Similarly, the terms "aralkenyl" and "heteroaralkenyl" refer to an aryl group or a heteroaryl group, respectively bonded directly through an alkene group, such as benzyl. Aralkenyl and heteroaralkenyl may be optionally substituted as the aryl and heteroaryl groups.

The compounds of the present invention can possess one or more asymmetric carbon atoms and can exist as optical isomers forming mixtures of racemic or non-racemic compounds. The compounds of the present invention are useful as single isomers or as a mixture of stereochemical isomeric forms. Diastereoisomers, i.e., nonsuperimposable stereochemical isomers, can be separated by conventional means such as chromatography, distillation, crystallization or sublimation. The optical isomers can be obtained by resolution of the racemic mixtures according to conventional processes.

The invention encompasses isolated or purified compounds. An "isolated" or "purified" compound refers to a compound which represents at least 10%, 20%, 50%, 80% or 90% of the compound of the present invention present in a mixture, provided that the mixture comprising the compound of the invention has demonstrable (i.e. statistically significant) biological activity including antibacterial, cytostatic, cytotoxic, antiinflammatory or enzyme inhibitory action when tested in conventional biological assays known to a person skilled in the art.

The terms "farnesyl dibenzodiazepinone-producing microorganism" and "producer of farnesyl dibenzodiazepinone," as used herein, refer to a microorganism that carries genetic information necessary to produce a farnesyl dibenzodiazepinone compound, whether or not the organism naturally produces the compound. The terms apply equally to organisms in which the genetic information to produce the farnesyl

dibenzodiazepinone compound is found in the organism as it exists in its natural environment, and to organisms in which the genetic information is introduced by recombinant techniques.

Specific organisms contemplated herein include, without limitation, organisms of the family Micromonosporaceae, of which preferred genera include Micromonospora, Actinoplanes and Dactylosporangium; the family Streptomycetaceae, of which preferred genera include Streptomyces and Kitasatospora; the family Pseudonocardiaceae, of which preferred genera are Amycolatopsis and Saccharopolyspora; and the family Actinosynnemataceae, of which preferred genera include Saccharothrix and Actinosynnema; however the terms are intended to encompass all organisms containing genetic information necessary to produce a farnesyl dibenzodiazepinone compound. A preferred producer of a farnesyl dibenzodiazepinone compound includes microbial strain 046-ECO11, a deposit of which was made on March 7, 2003, with the International Depository Authority of Canada (IDAC), Bureau of Microbiology, Health Canada, 1015 Arlington Street, Winnipeg, Manitoba, Canada R3E 3R2, under Accession No. IDAC 070303-01.

The term "gene" means the segment of DNA involved in producing a polypeptide chain; it includes regions preceding and following the coding region (leader and trailer) as well as, where applicable, intervening regions (introns) between individual coding segments (exons).

The terms "gene locus, "gene cluster," and "biosynthetic locus" refer to a group of genes or variants thereof involved in the biosynthesis of a farnesyl benzodiazepinone compound. The biosynthetic locus in strain 046-ECO11 that directs the production of ECO-04601 is often referred to herein, in both the written description and Figures, as "046D." Genetic modification of gene locus, gene cluster or biosynthetic locus refers to any genetic recombinant techniques known in the art including mutagenesis, inactivation, or replacement of nucleic acids that can be applied to generate variants of ECO-04601.

A DNA or nucleotide "coding sequence" or "sequence encoding" a particular polypeptide or protein, is a DNA sequence which is transcribed and translated into a

polypeptide or protein when placed under the control of an appropriate regulatory sequence.

"Oligonucleotide" refers to a nucleic acid, generally of at least 10, preferably 15 and more preferably at least 20 nucleotides in length, preferably no more than 100 nucleotides in length, that are hybridizable to a genomic DNA molecule, a cDNA molecule, or an mRNA molecule encoding a gene, mRNA, cDNA or other nucleic acid of interest.

A promoter sequence is "operably linked to" a coding sequence recognized by RNA polymerase which initiates transcription at the promoter and transcribes the coding sequence into mRNA.

The term "replicon" as used herein means any genetic element, such as a plasmid, cosmid, chromosome or virus, that behaves as an autonomous unit of polynucleotide replication within a cell. A "expression vector" or "vector" is a replicon in which another polynucleotide fragment is attached, such as to bring about the replication and/or expression of the attached fragment. "Plasmids" are designated herein by a lower case "p" preceded or followed by capital letters and/or numbers. The starting plasmids disclosed herein are commercially available, publicly available on an unrestricted basis, or can be constructed from available plasmids in accordance with published procedures. In addition, equivalent plasmids to those described herein are known in the art and will be apparent to the skilled artisan.

The terms "express" and "expression" means allowing or causing the information in a gene or DNA sequence to become manifest, for example producing a protein by activating the cellular functions involved in transcription and translation of a corresponding gene or DNA sequence. A DNA sequence is expressed in or by a cell to form an "expression product" such as a protein. The expression product itself, e.g. the resulting protein, may also be said to be "expressed" by the cell. An expression product can be characterized as intracellular, extracellular or secreted.

"Digestion" of DNA refers to enzymatic cleavage of the DNA with a restriction enzyme that acts only at certain sequences in the DNA. The various restriction enzymes used herein are commercially available and their reaction conditions,

cofactors and other requirements were used as would be known to the ordinary skilled artisan. For analytical purposes, typically 1 µg of plasmid or DNA fragment is used with about 2 units of enzyme in about 20 µl of buffer solution. For the purpose of isolating DNA fragments for plasmid construction, typically 5 to 50 µg of DNA are digested with 20 to 250 units of enzyme in a larger volume. Appropriate buffers and substrate amounts for particular enzymes are specified by the manufacturer. Incubation times of about 1 hour at 37°C are ordinarily used, but may vary in accordance with the supplier's instructions. After digestion the gel electrophoresis may be performed to isolate the desired fragment.

The term "isolated" as used herein means that the material is removed from its original environment (e.g. the natural environment where the material is naturally occurring). For example, a naturally occurring polynucleotide or polypeptide present in a living animal is not isolated, but the same polynucleotide or polypeptide, which is separated from some or all of the coexisting materials in the natural system, is isolated. Such polynucleotides could be part of a vector and/or such polynucleotides or polypeptides could be part of a composition, and still be isolated in that the vector or composition is not part of the natural environment.

The term "restriction fragment" as used herein refers to any linear DNA generated by the action of one or more restriction enzymes.

The term "transformation" means the introduction of a foreign gene, foreign nucleic acid, DNA or RNA sequence to a host cell, so that the host cell will express the introduced gene or sequence to produce a desired substance, typically a protein or enzyme coded by the introduced gene or sequence. The introduced gene or sequence may also be called a "cloned" or "foreign" gene or sequence, may include regulatory or control sequences, such as start, stop, promoter, signal, secretion, or other sequences used by a cell's genetic machinery. The gene or sequence may include nonfunctional sequences or sequences with no known function. A host cell that receives and expresses introduced DNA or RNA has been "transformed" and is a "transformant" or a "clone" or "recombinant". The DNA or RNA introduced to a host cell can come from any source, including cells of the same genus or species as the host cell, or cells of a

different genus or species.

The terms "recombinant polynucleotide" and "recombinant polypeptide" as used herein mean a polynucleotide or polypeptide which by virtue of its origin or manipulation is not associated with all or a portion of the polynucleotide or polypeptide with which it is associated in nature and/or is linked to a polynucleotide or polypeptide other than that to which it is linked in nature.

The term "host cell" as used herein, refer to both prokaryotic and eukaryotic cells which are used as recipients of the recombinant polynucleotides and vectors provided herein. In one embodiment, the host cell is a prokaryote.

The terms "open reading frame" and "ORF" as used herein refers to a region of a polynucleotide sequence which encodes a polypeptide; this region may represent a portion of a coding sequence or a total coding sequence.

As used herein and as known in the art, the term "identity" is the relationship between two or more polynucleotide sequences, as determined by comparing the sequences. Identity also means the degree of sequence relatedness between polynucleotide sequences, as determined by the match between strings of such sequences. Identity can be readily calculated (see, e.g., *Computation Molecular Biology*, Lesk, A.M., eds., Oxford University Press, New York (1998), and *Biocomputing: Informatics and Genome Projects*, Smith, D.W., ed., Academic Press, New York (1993), both of which are incorporated by reference herein). While there exist a number of methods to measure identity between two polynucleotide sequences, the term is well known to skilled artisans (see, e.g., *Sequence Analysis in Molecular Biology*, von Heinje, G., Academic Press (1987); and *Sequence Analysis Primer*, Gribskov., M. and Devereux, J., eds., M. Stockton Press, New York (1991)). Methods commonly employed to determine identity between sequences include, for example, those disclosed in Carillo, H., and Lipman, D., *SIAM J. Applied Math.* (1988) **48**:1073. "Substantially identical," as used herein, means there is a very high degree of homology (preferably 100% sequence identity) between subject polynucleotide sequences. However, polynucleotides having greater than 90%, or 95% sequence identity may be

used in the present invention, and thus sequence variations that might be expected due to genetic mutation, strain polymorphism, or evolutionary divergence can be tolerated.

As used herein, the term "treatment" refers to the application or administration of a therapeutic agent to a patient, or application or administration of a therapeutic agent to an isolated tissue or cell line from a patient, who has a disorder, e.g., a disease or condition, a symptom of disease, or a predisposition toward a disease, with the purpose to cure, heal, alleviate, relieve, alter, remedy, ameliorate, improve, or affect the disease, the symptoms of disease, or the predisposition toward disease.

As used herein, a "pharmaceutical composition" comprises a pharmacologically effective amount of a farnesyl dibenzodiazepinone and a pharmaceutically acceptable carrier. As used herein, "pharmacologically effective amount," "therapeutically effective amount" or simply "effective amount" refers to that amount of a farnesyl dibenzodiazepinone effective to produce the intended pharmacological, therapeutic or preventive result. For example, if a given clinical treatment is considered effective when there is at least a 25% reduction in a measurable parameter associated with a disease or disorder, a therapeutically effective amount of a drug for the treatment of that disease or disorder is the amount necessary to effect at least a 25% reduction in that parameter.

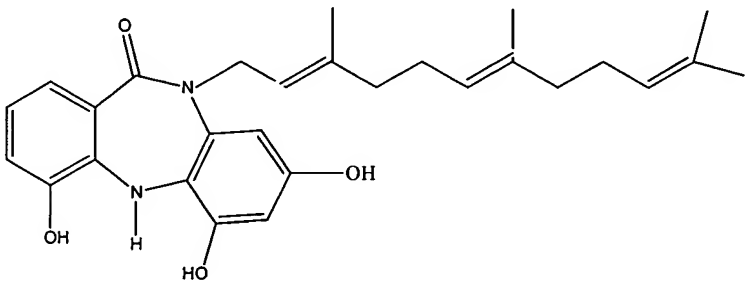
The term "pharmaceutically acceptable carrier" refers to a carrier for administration of a therapeutic agent. Such carriers include, but are not limited to, saline, buffered saline, dextrose, water, glycerol, ethanol, and combinations thereof. The term specifically excludes cell culture medium. For drugs administered orally, pharmaceutically acceptable carriers include, but are not limited to pharmaceutically acceptable excipients such as inert diluents, disintegrating agents, binding agents, lubricating agents, sweetening agents, flavoring agents, coloring agents and preservatives. Suitable inert diluents include sodium and calcium carbonate, sodium and calcium phosphate, and lactose, while corn starch and alginic acid are suitable disintegrating agents. Binding agents may include starch and gelatin, while the lubricating agent, if present, will generally be magnesium stearate, stearic acid or talc. If desired, the tablets may be coated with a material such as glyceryl monostearate or

glyceryl distearate, to delay absorption in the gastrointestinal tract.

The term "pharmaceutically acceptable salt" refers to both acid addition salts and base addition salts. The nature of the salt is not critical, provided that it is pharmaceutically acceptable. Exemplary acid addition salts include, without limitation, hydrochloric, hydrobromic, hydroiodic, nitric, carbonic, sulphuric, phosphoric, formic, acetic, citric, tartaric, succinic, oxalic, malic, glutamic, propionic, glycolic, gluconic, maleic, embonic (pamoic), methanesulfonic, ethanesulfonic, 2-hydroxyethanesulfonic, pantothenic, benzenesulfonic, toluenesulfonic, sulfanilic, mesylic, cyclohexylaminosulfonic, stearic, algenic, β-hydroxybutyric, malonic, galactaric, galacturonic acid and the like. Suitable pharmaceutically acceptable base addition salts include, without limitation, metallic salts made from aluminium, calcium, lithium, magnesium, potassium, sodium and zinc or organic salts made from N,N'-dibenzylethylenediamine, chloroprocaine, choline, diethanolamine, ethylenediamine, N-methylglucamine, lysine, procaine and the like. Additional examples of pharmaceutically acceptable salts are listed *in Journal of Pharmaceutical Sciences* (1977) 66:2. All of these salts may be prepared by conventional means from a farnesyl dibenzodiazepinone by treating the compound with the appropriate acid or base.

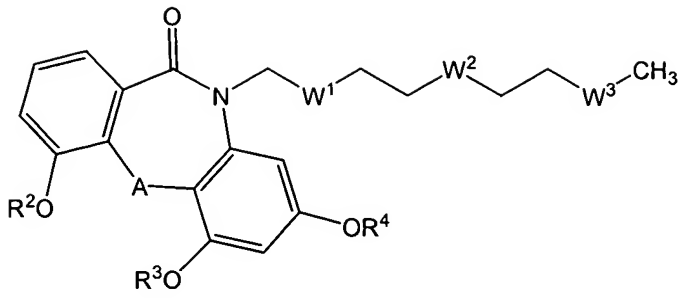
II. Farnesylated Dibenzodiazepinone Compounds

In one aspect, the invention relates to a novel farnesyl dibenzodiazepinone, referred to herein as "ECO-04601" and having the chemical structure represented by the following formula:



ECO-04601 may be described as a new dibenzodiazepinone having a 10-farnesyl substituent located on the nitrogen atom in the 10 position of the dibenzodiazepine ring (i.e., the amide nitrogen in the diazepinone ring), and three phenolic hydroxy substituents in the 4,6 and 8 positions of the dibenzodiazepinone ring. ECO-04601 may be characterized by any one or more of its physicochemical and spectral properties given below, such as its mass, UV, and NMR spectroscopic data. Mass was determined by electrospray mass spectrometry to be 462.6 (FIGURE 1); UV = 230 nm with a shoulder at 290nm (FIGURE 2). NMR data were collected using MeOH- *d4*, including proton (FIGURE 3), and multidimensional pulse sequences gDQCOSY (FIGURE 4), gHSQC (FIGURE 5), gHMBC (FIGURE 6), and NOESY (FIGURE 7).

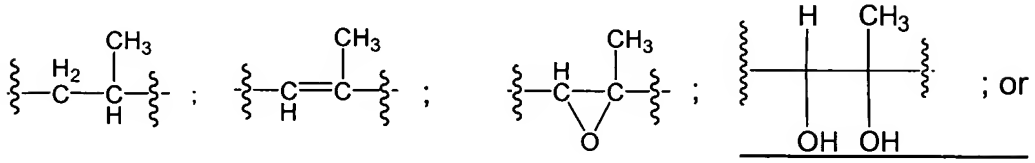
In another aspect, the invention relates to a novel class of farnesyl dibenzodiazepinone compounds represented by Formula I:



Formula I.

wherein,

W1, W2 and W3 is each independently selected from



the chain from the tricycle may terminate at W3, W2 or W1 with W3, W2 or W1 respectively being either –CH=O or –CH2OH;

A is selected from –NH--, –NCH2R1, -NC(O)R1;

R1 is selected from C1-6 alkyl, C2-6 alkene, aryl or heteroaryl;

R2, R3, and R4 is each independently selected from H, R5, -C(O)R6

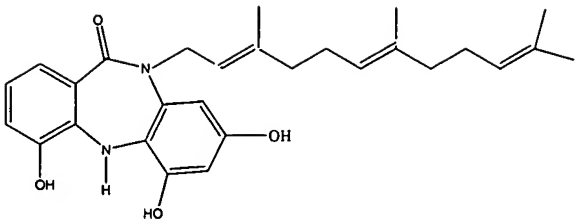
R5 is each independently selected from C_{1-6} alkyl, C_{2-7} alkalene, aryl or heteroaryl;

R6 is each independently selected from H, C_{1-6} alkyl, C_{2-7} alkalene, aryl or heteroaryl; or a pharmaceutically acceptable salt thereof.

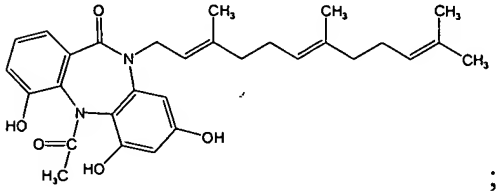
In other embodiments, the invention provides compounds of Formula I, wherein A is selected from the group consisting of NH, NCH2R1, and NC(O)R1; wherein R2 is H; R3 is H; and R4 is H. In another embodiment, R2, R3 and R4 are each H; and all other groups are as previously defined. In a further embodiment, R2, R3 and R4 are each H; and W1 is –CH =CH- and all other groups are as previously defined. In a

further embodiment, R2, R3 and R4 are each H, and W2 is –CH =CH- and all other groups are as previously defined. In a further embodiment, R2, R3 and R4 are each H; and W3 is –CH =CH- ; and all other groups are as previously defined. In a further embodiment, A is NH; R2, R3 and R4 are each H; and all other groups are as previously defined. In a further embodiment, A is NH; each of W1, W2, and W3 is –CH =CH-; and all other groups are as previously defined. The invention encompasses all pharmaceutically acceptable salts of the foregoing compounds.

The following are exemplary compounds of the invention:



Formula II



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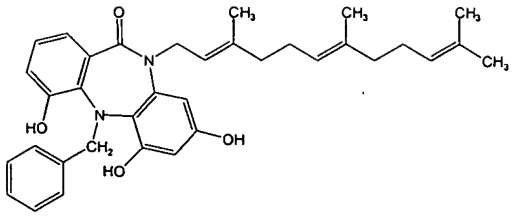
Formula III



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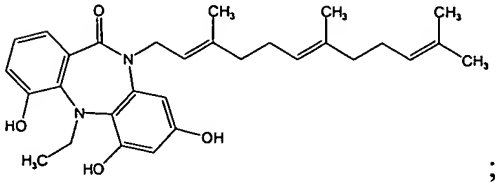

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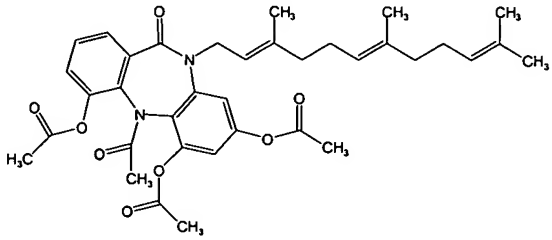
Formula V



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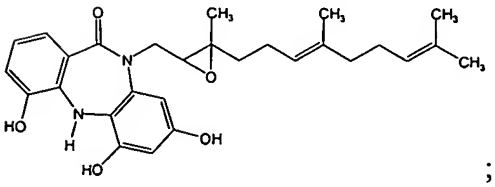

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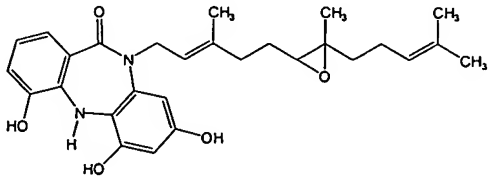

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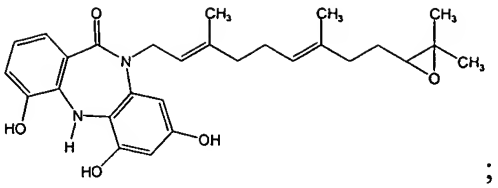

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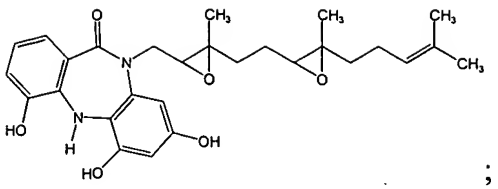
Formula VIX



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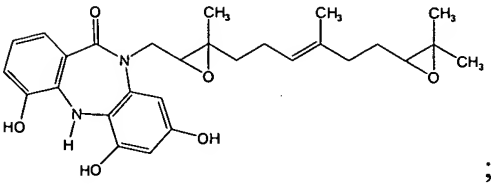
Formula X



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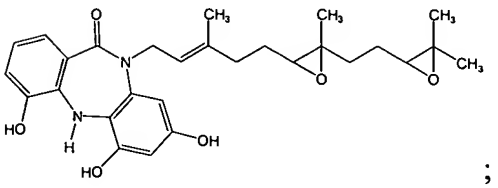

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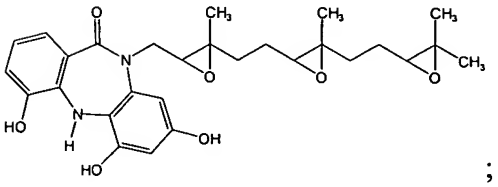

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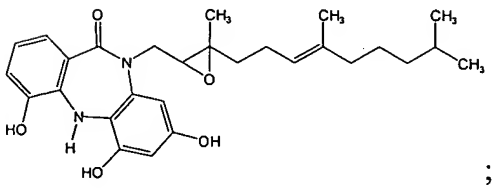

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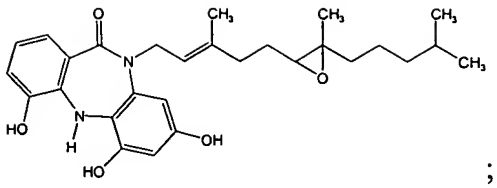

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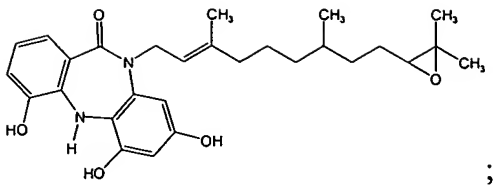

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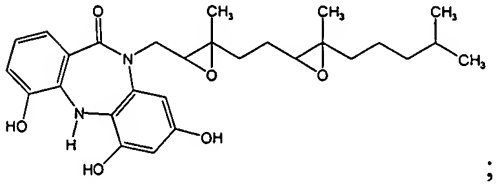

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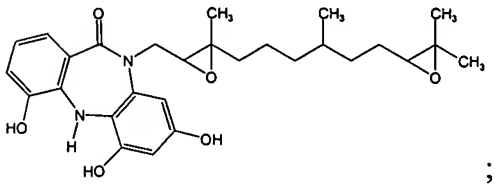

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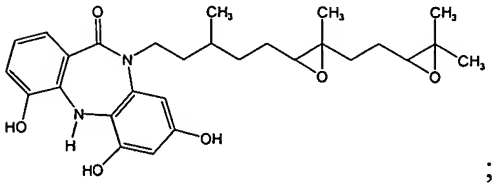

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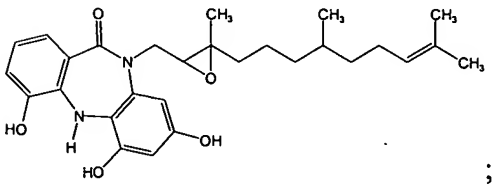

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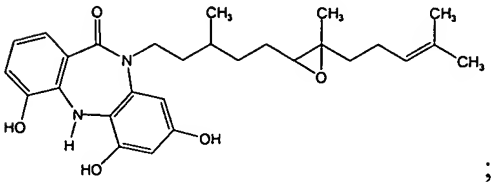
Formula XX



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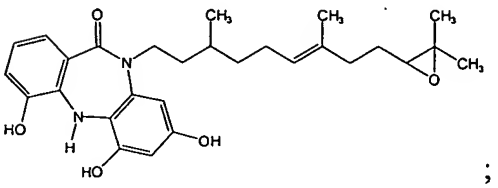

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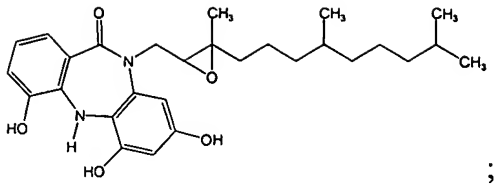

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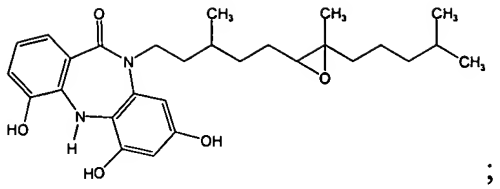

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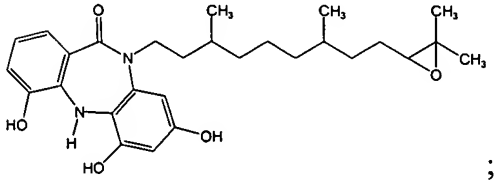

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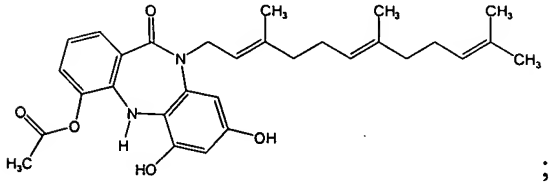

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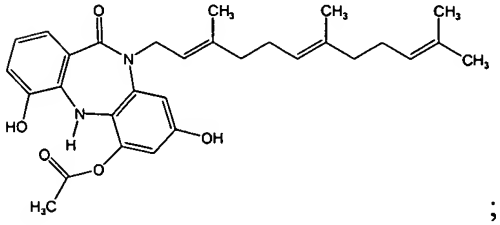

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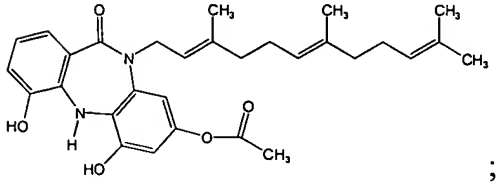

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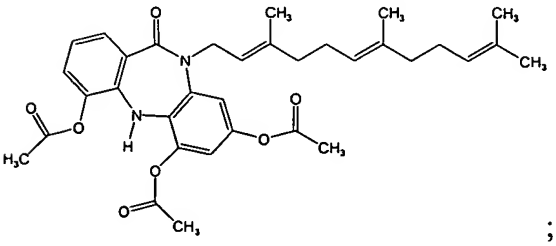

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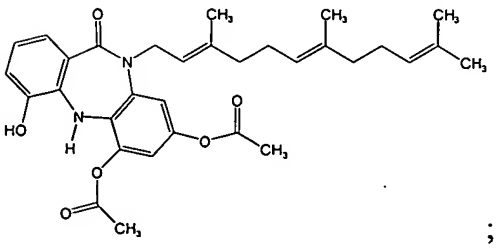

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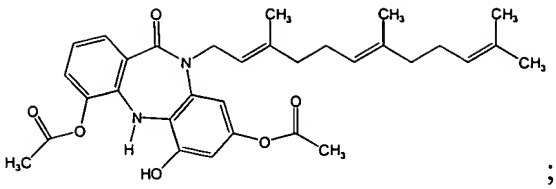
Formula XXX



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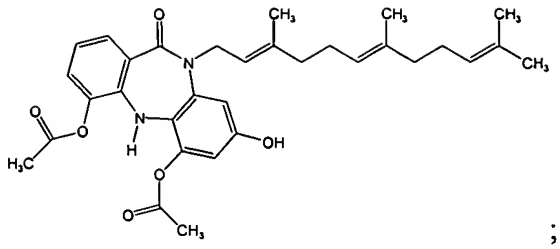

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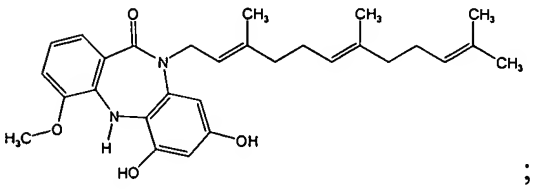

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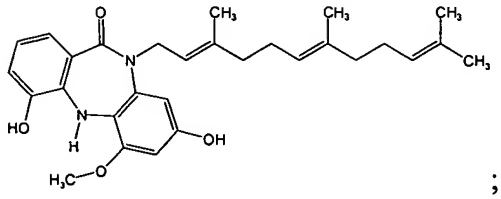

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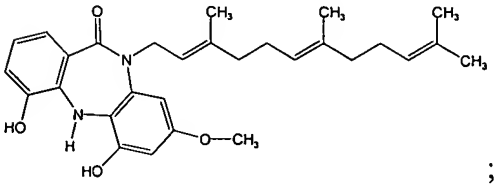

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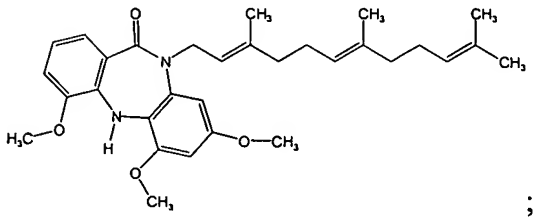

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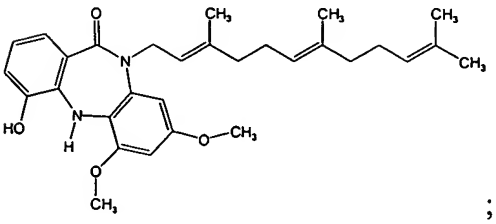

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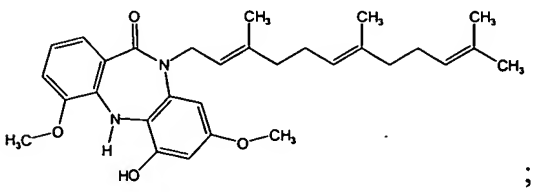

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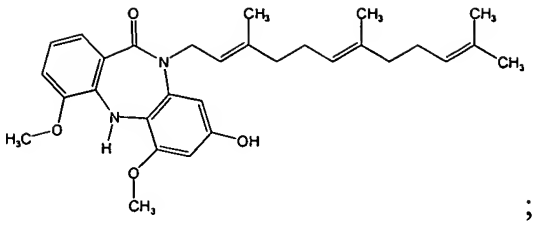

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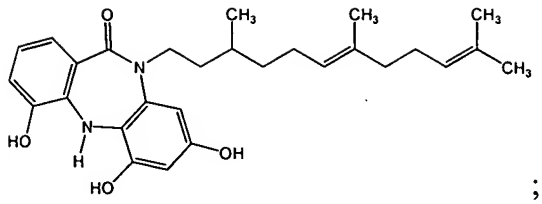

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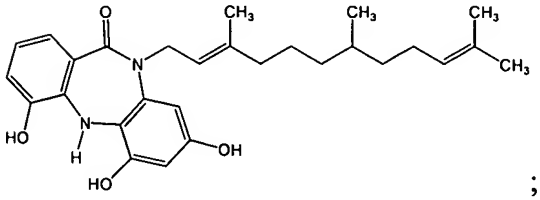

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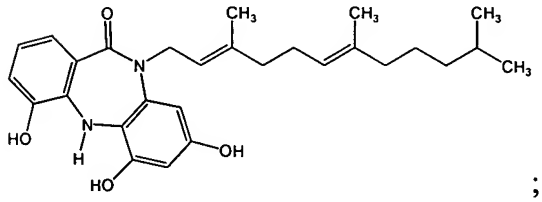

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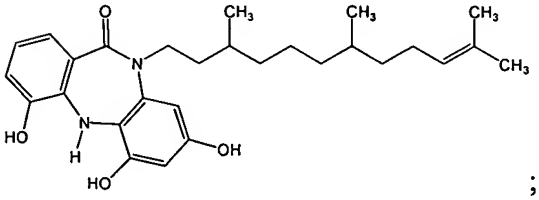

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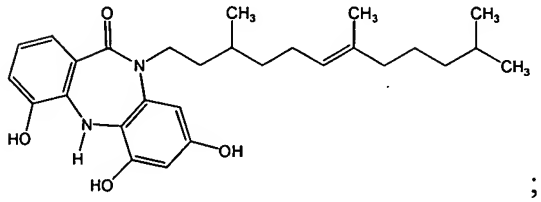

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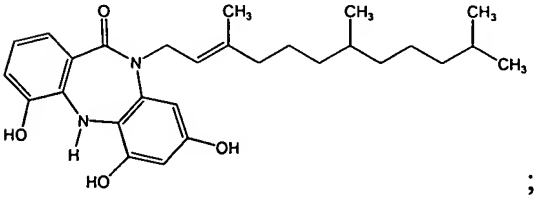

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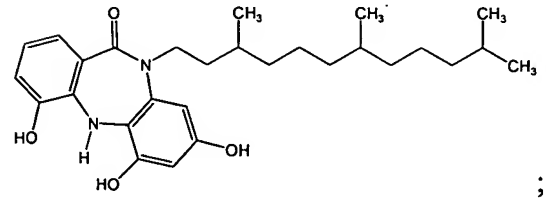

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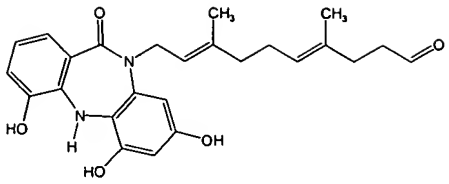

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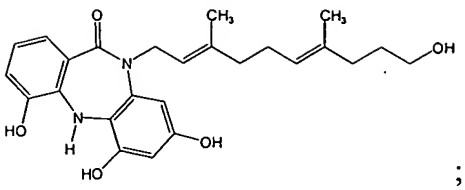

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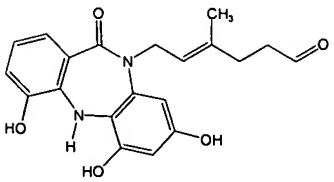

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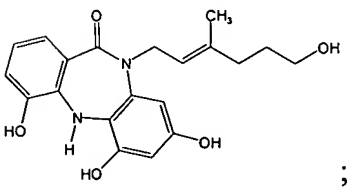

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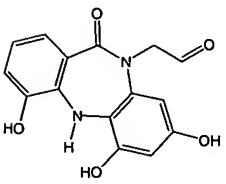

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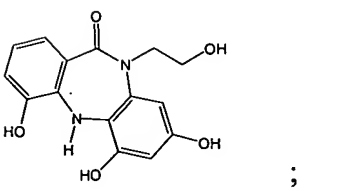

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Formula LI

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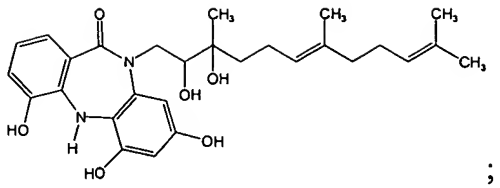

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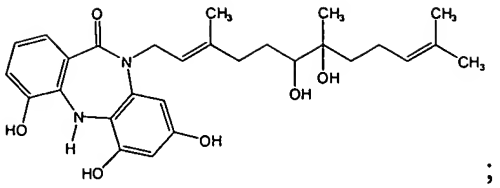

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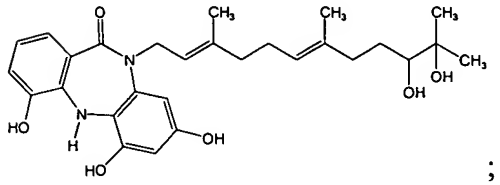

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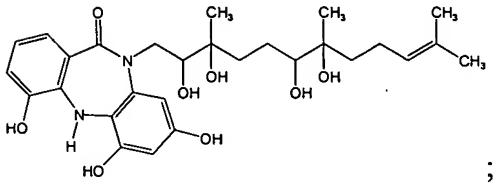

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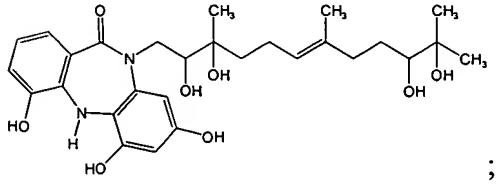

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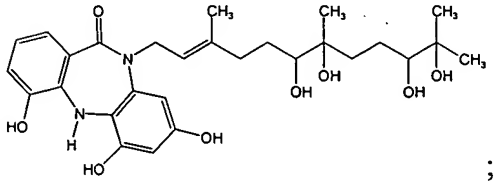

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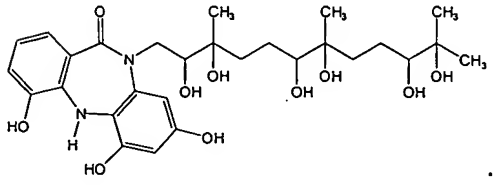
Formula LVIII



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Formula LIX

Certain embodiments expressly exclude one or more of the compounds of Formula I. In one embodiment, the compound of Formula II is excluded.

The compounds of this invention may be formulated into pharmaceutical compositions comprised of compounds of Formula I in combination with a

pharmaceutical acceptable carrier, as discussed in Section V below.

III. Method of Making a Farnesyl Dibenzodiazepinone by Fermentation

In one embodiment, ECO-04601 is obtained by cultivating a novel strain of Micromonospora, namely *Micromonospora sp.* strain 046-ECO11. Strain 046-ECO11 was deposited on March 7, 2003, with the International Depositary Authority of Canada (IDAC), Bureau of Microbiology, Health Canada, 1015 Arlington Street, Winnipeg, Manitoba, Canada R3E 3R2, under Accession No. 070303-01. The deposit of the strain was made under the terms of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for Purposes of Patent Procedure. The deposited strains will be irrevocably and without restriction or condition released to the public upon the issuance of a patent. The deposited strains are provided merely as convenience to those skilled in the art and are not an admission that a deposit is required for enablement, such as that required under 35 U.S.C. §112.

It is to be understood that the present invention is not limited to use of the particular strain 046-ECO11. Rather, the present invention contemplates the use of other ECO-04601 producing organisms, such as mutants or variants of 046-ECO11 that can be derived from this organism by known means such as X-ray irradiation, ultraviolet irradiation, treatment with nitrogen mustard, phage exposure, antibiotic selection and the like; or through the use of recombinant genetic engineering techniques, as described in Section IV below.

The farnesyl dibenzodiazepinone compounds of the present invention may be biosynthesized by various microorganisms. Microorganisms that may synthesize the compounds of the present invention include but are not limited to bacteria of the order Actinomycetales, also referred to as actinomycetes. Non-limiting examples of members belonging to the genera of Actinomycetes include Nocardia, Geodermatophilus, Actinoplanes, Micromonospora, Nocardioides, Saccharothrix, Amycolatopsis, Kutzneria, Saccharomonospora, Saccharopolyspora, Kitasatospora, Streptomyces, Microbispora, Streptosporangium, and Actinomadura. The taxonomy of actinomycetes is complex

and reference is made to Goodfellow, *Suprageneric Classification of Actinomycetes* (1989); *Bergey's Manual of Systematic Bacteriology*, Vol. 4 (Williams and Wilkins, Baltimore, pp. 2322-2339); and to Embley and Stackebrandt, "The molecular phylogeny and systematics of the actinomycetes," *Annu. Rev. Microbiol.* (1994) 48:257-289, each of which is hereby incorporated by reference in its entirety, for genera that may synthesize the compounds of the invention.

Farnesyl dibenzodiazepinone-producing microorganisms are cultivated in culture medium containing known nutritional sources for actinomycetes. Such media having assimilable sources of carbon, nitrogen, plus optional inorganic salts and other known growth factors at a pH of about 6 to about 9. Suitable media include, without limitation, the growth media provided in Table 16. Microorganisms are cultivated at incubation temperatures of about 18 °C to about 40 °C for about 3 to about 40 days.

The culture media inoculated with the farnesyl dibenzodiazepinone-producing microorganisms may be aerated by incubating the inoculated culture media with agitation, for example, shaking on a rotary shaker, or a shaking water bath. Aeration may also be achieved by the injection of air, oxygen or an appropriate gaseous mixture to the inoculated culture media during incubation. Following cultivation, the farnesyl dibenzodiazepinone compounds can be extracted and isolated from the cultivated culture media by techniques known to a skilled person in the art and/or disclosed herein, including for example centrifugation, chromatography, adsorption, filtration. For example, the cultivated culture media can be mixed with a suitable organic solvent such as n-butanol, n-butyl acetate or 4-methyl-2-pentanone, the organic layer can be separated for example, by centrifugation followed by the removal of the solvent, by evaporation to dryness or by evaporation to dryness under vacuum. The resulting residue can optionally be reconstituted with for example water, ethanol, ethyl acetate, methanol or a mixture thereof, and re-extracted with a suitable organic solvent such as hexane, carbon tetrachloride, methylene chloride or a mixture thereof. Following removal of the solvent, the compounds may be further purified by the use of standard techniques, such as chromatography.

The farnesyl dibenzodiapezinones biosynthesized by microorganisms may optionally be subjected to random and/or directed chemical modifications to form compounds that are derivatives or structural analogs. Such derivatives or structural analogs having similar functional activities are within the scope of the present invention. Farnesyl dibenzodiapezinone compounds may optionally be modified using methods known in the art and described herein.

IV. Method of Making a Farnesyl Dibenzodiazepinone by Recombinant Technology

In another embodiment, the present invention relates to nucleic acid molecules that encode proteins useful in the production of farnesyl benzodiazepinones. Specifically, the present invention provides recombinant DNA vectors and nucleic acid molecules that encode all or part of the biosynthetic locus in strain 046-ECO11, which directs the production of ECO-04601, and is referred to herein as "046D." The invention further includes genetic modification of 046D using conventional genetic recombinant techniques, such as mutagenesis, inactivation, or replacement of nucleic acids, to produce chemical variants of ECO-04601.

The invention thus provides a method for making a farnesyl benzodiazepinone compound using a transformed host cell comprising a recombinant DNA vector that encodes one or more of the polypeptides of the present invention, and culturing the host cell under conditions such that farnesyl benzodiazepinone is produced. The host cell is a prokaryote. In one embodiment, the host cell is an actinomycete. In another embodiment, the host cell is a *Streptomyces* host cell.

The invention provides recombinant nucleic acids that produce a variety of farnesyl dibenzodiazepinone compounds that cannot be readily synthesized by chemical methodology alone. The invention allows direct manipulation of 046D biosynthetic locus via genetic engineering of the enzymes involved in the biosynthesis of a farnesyl benzodiazepinone according to the invention. The 046A biosynthetic locus is described in Example 11.

Recombinant DNA Vectors

Vectors of the invention typically comprise the DNA of a transmissible agent, into

which foreign DNA is inserted. A common way to insert one segment of DNA into another segment of DNA involves the use of specific enzymes called restriction enzymes that cleave DNA at specific sites (specific groups of nucleotides) called restriction sites. A "cassette" refers to a DNA coding sequence or segment of DNA that codes for an expression product that can be inserted into a vector at defined restriction sites. The cassette restriction sites are designed to ensure insertion of the cassette in the proper reading frame. Generally, a nucleic acid molecule that encodes a protein useful in the production of a farnesyl benzodiazepinone is inserted at one or more restriction sites of the vector DNA, and then is carried by the vector into a prokaryote e.g. actinomycte, by transformation (see below). A segment or sequence of DNA having inserted or added DNA, such as an expression vector, can also be called a "DNA construct". A common type of vector is a "plasmid" which generally is a self-contained molecule of double-stranded DNA, usually of bacterial origin, that can readily accept additional (foreign) DNA and which can be readily introduced into a suitable host cell. A plasmid vector often contains coding DNA and promoter DNA and has one or more restriction sites suitable for inserting foreign DNA. Coding DNA is a DNA sequence that encodes a particular amino acid sequence for a particular protein or enzyme. In one embodiment of the invention, the coding DNA encodes for polypeptides of SEQ ID NOs. 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 66, 68, 70, 72, 75, 77, 79, 81, 83, 85, 87 or 89 that are required for the biosynthesis of a farnesyl benzodiazepinone.

Promoter DNA of a recombinant vector is a DNA sequence that initiates, regulates, or otherwise mediates or controls the expression of the coding DNA. Promoter DNA and coding may be from the same or different organisms. Recombinant cloning vectors will often include one or more replication systems for cloning or expression, one or more markers for selection in the host, e.g. antibiotic resistance, and one or more expression cassettes. Vector constructs may be produced using conventional molecular biology and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Sambrook, Fritsch & Maniatis, Molecular Cloning: A Laboratory Manual, Second Edition (1989) Cold Spring

Harbor Laboratory Press, Cold Spring Harbor, New York (herein "Sambrook et al., 1989"); DNA Cloning: A Practical Approach, Volumes I and II (D. N. Glover ed. 1985); F. M. Ausubel et al. (eds.), Current Protocols in Molecular Biology, John Wiley & Sons, Inc. (1994).

Examples of promoters that function in actinomycetes, e.g. Streptomyces, are taught in US Patent Nos. 5,830,695 and 5,466,590. Another example of a transcription promoter useful in Actinomycetes expression vectors is tipA, a promoter inducible by the antibiotic thiostrepton [c.f. Murakami, T., et al., (1989), J. Bacteriol. , 171, 1459].

Transformation of Actinomycetes

A suitable transformation method for use with an actinomycete comprises forming the actinomycete culture into spheroplasts using lysozyme. A buffer solution containing recombinant DNA vectors and polyethylene glycol is then added, in order to introduce the vector into the host cells, by using either of the methods of Thompson or Keiser [c. f. Thompson, C. J., et al., (1982), J. Bacteriol., 151, 668-677 or Keiser, T. et al. (2000), "Practical Streptomyces Genetics", The John Innes Foundation, Norwich], for example. A thiostrepton-resistance gene is frequently used as a selective marker in the transformation plasmid [c.f. Hopwood, D. A., et al., (1987), "Methods in Enzymology" 153, 116, Academic Press, New York], but the present invention is not limited thereto. Additional methods for the transformation of actinomycetes are taught in US 5,393,665.

Assay for farnesyl dibenzodiazepinone or biosynthetic intermediates

Actinomycetes defective in farnesyl dibenzodiazepinone biosynthesis are transformed with one or more expression vectors encoding one or more proteins in the farnesyl benzodiazepinone biosynthetic pathway, thus restoring farnesyl benzodiazepinone biosynthesis by genetic complementation of the specific defect.

The presence or absence of farnesyl dibenzodiazepinone or intermediates in the biosynthetic pathway (see Figures 13, 14 and 15) in a recombinant actinomycete can be determined using methodologies that are well known to persons of skill in the art.

For example, ethyl acetate extracts of fermentation media used for the culture of a recombinant actinomycete are processed as described in Example 2 and fractions containing farnesyl dibenzodiazepinone or intermediates detected by TLC on commercial Kieselgel $60F_{254}$ plates. Farnesyl dibenzodiazepinone and intermediate compounds are visualized by inspection of dried plates under UV light or by spraying the plates with a spray containing vanillin (0.75%) and concentrated sulfuric acid (1.5%, v/v) in ethanol and subsequently heating the plate. The exact identity of the compounds separated by TLC is then determined using gas chromatography-mass spectroscopy. Methods of mass spectroscopy are taught in the published U.S. Patent Application No. US2003/0052268.

Mutagenesis

The invention allows direct manipulation of 046D biosynthetic locus via genetic engineering of the enzymes involved in the biosynthesis of a farnesyl benzodiazepinone according to the invention.

A number of methods are known in the art that permit the random as well as targeted mutation of the DNA sequences of the invention (see for example, Ausubel et. al. Short Protocols in Molecular Biology (1995) 3rd Ed. John Wiley & Sons, Inc.). In addition, there are a number of of commercially available kits for site-directed mutagenesis, including both conventional and PCR-based methods. Examples include the EXSITE™ PCR-Based Site-directed Mutagenesis Kit available from Stratagene (Catalog No. 200502) and the QUIKCHANGE™ Site-directed mutagenesis Kit from Stratagene (Catalog No. 200518), and the CHAMELEON® double-stranded Site-directed mutagenesis kit, also from Stratagene (Catalog No. 200509).

In addition the nucleotides of the invention may be generated by insertional mutation or truncation (N-terminal, internal or C-terminal) according to methodology known to a person skilled in the art.

Older methods of site-directed mutagenesis known in the art rely on sub-cloning of the sequence to be mutated into a vector, such as an M13 bacteriophage vector, that allows the isolation of single-stranded DNA template. In these methods, one anneals a mutagenic primer (i.e., a primer capable of annealing to the site to be mutated but

bearing one or more mismatched nucleotides at the site to be mutated) to the single-stranded template and then polymerizes the complement of the template starting from the 3' end of the mutagenic primer. The resulting duplexes are then transformed into host bacteria and plaques are screened for the desired mutation.

More recently, site-directed mutagenesis has employed PCR methodologies, which have the advantage of not requiring a single-stranded template. In addition, methods have been developed that do not require sub-cloning. Several issues must be considered when PCR-based site-directed mutagenesis is performed. First, in these methods it is desirable to reduce the number of PCR cycles to prevent expansion of undesired mutations introduced by the polymerase. Second, a selection must be employed in order to reduce the number of non-mutated parental molecules persisting in the reaction. Third, an extended-length PCR method is preferred in order to allow the use of a single PCR primer set. And fourth, because of the non-template-dependent terminal extension activity of some thermostable polymerases it is often necessary to incorporate an end-polishing step into the procedure prior to blunt-end ligation of the PCR-generated mutant product.

The protocol described below accommodates these considerations through the following steps. First, the template concentration used is approximately 1000-fold higher than that used in conventional PCR reactions, allowing a reduction in the number of cycles from 25-30 down to 5-10 without dramatically reducing product yield. Second, the restriction endonuclease Dpn I (recognition target sequence: 5-Gm6ATC-3, where the A residue is methylated) is used to select against parental DNA, since most common strains of E. coli Dam methylate their DNA at the sequence 5-GATC-3. Third, Taq Extender is used in the PCR mix in order to increase the proportion of long (i.e., full plasmid length) PCR products. Finally, Pfu DNA polymerase is used to polish the ends of the PCR product prior to intramolecular ligation using T4 DNA ligase.

A non-limiting example for the isolation of mutant polynucleotides is described in detail as follows:

Plasmid template DNA (approximately 0.5 pmole) is added to a PCR cocktail containing: 1x mutagenesis buffer (20 mM Tris HCl, pH 7.5; 8 mM MgCl2; 40□g/ml

BSA); 12-20 pmole of each primer (one of skill in the art may design a mutagenic primer as necessary, giving consideration to those factors such as base composition, primer length and intended buffer salt concentrations that affect the annealing characteristics of oligonucleotide primers; one primer must contain the desired mutation, and one (the same or the other) must contain a 5' phosphate to facilitate later ligation), 250 □M each dNTP, 2.5 U Taq DNA polymerase, and 2.5 U of Taq Extender (Available from Stratagene; See Nielson et al. (1994) Strategies 7: 27, and U.S. Patent No. 5,556,772). Primers can be prepared using the triester method of Matteucci et al., 1981, J. Am. Chem. Soc. 103:3185-3191, incorporated herein by reference. Alternatively automated synthesis may be preferred, for example, on a Biosearch 8700 DNA Synthesizer using cyanoethyl phosphoramidite chemistry.

The PCR cycling is performed as follows: 1 cycle of 4 min at 94oC, 2 min at 50oC and 2 min at 72oC; followed by 5-10 cycles of 1 min at 94oC, 2 min at 54oC and 1 min at 72oC. The parental template DNA and the linear, PCR-generated DNA incorporating the mutagenic primer are treated with DpnI (10 U) and Pfu DNA polymerase (2.5U). This results in the DpnI digestion of the in vivo methylated parental template and hybrid DNA and the removal, by Pfu DNA polymerase, of the non-template-directed Taq DNA polymerase-extended base(s) on the linear PCR product. The reaction is incubated at 37oC for 30 min and then transferred to 72oC for an additional 30 min. Mutagenesis buffer (115 ul of 1x) containing 0.5 mM ATP is added to the DpnI-digested, Pfu DNA polymerase-polished PCR products. The solution is mixed and 10 ul are removed to a new microfuge tube and T4 DNA ligase (2-4 U) is added. The ligation is incubated for greater than 60 min at 37oC. Finally, the treated solution is transformed into competent E. coli according to standard methods.

Methods of random mutagenesis, which will result in a panel of mutants bearing one or more randomly situated mutations, exist in the art. Such a panel of mutants may then be screened for those exhibiting reduced uracil detection activity relative to the wild-type polymerase (e.g., by measuring the incorporation of 10nmoles of dNTPs into polymeric form in 30 minutes in the presence of 200□M dUTP and at the optimal temperature for a given DNA polymerase). An example of a method for random

mutagenesis is the so-called "error-prone PCR method". As the name implies, the method amplifies a given sequence under conditions in which the DNA polymerase does not support high fidelity incorporation. The conditions encouraging error-prone incorporation for different DNA polymerases vary, however one skilled in the art may determine such conditions for a given enzyme. A key variable for many DNA polymerases in the fidelity of amplification is, for example, the type and concentration of divalent metal ion in the buffer. The use of manganese ion and/or variation of the magnesium or manganese ion concentration may therefore be applied to influence the error rate of the polymerase.

Genes for desired mutant polypeptides generated by mutagenesis may be sequenced to identify the sites and number of mutations. For those mutants comprising more than one mutation, the effect of a given mutation may be evaluated by introduction of the identified mutation to the wild-type gene by site-directed mutagenesis in isolation from the other mutations borne by the particular mutant. Screening assays of the single mutant thus produced will then allow the determination of the effect of that mutation alone.

V. Genes and proteins for the production of ECO-04601

As discussed in more detail below, the isolated, purified or enriched nucleic acids of one of SEQ ID NOS: 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 66, 68, 70, 72, 75, 77, 79, 81, 83, 85, 87 and 89 may be used to prepare one of the polypeptides of SEQ ID NOS: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 41, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 65, 67, 69, 70, 71, 74, 76, 78, 80, 82, 84, 86 and 88, respectively, or fragments comprising at least 50, 75, 100, 200, 300, 500 or more consecutive amino acids of one of the polypeptides of SEQ ID NO: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 41, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 65, 67, 69, 70, 71, 74, 76, 78, 80, 82, 84, 86 and 88.

Accordingly, another aspect of the present invention is an isolated, purified or enriched nucleic acid which encodes one of the polypeptides of SEQ ID NOS: 2, 4, 6, 8,

10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 41, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 65, 67, 69, 70, 71, 74, 76, 78, 80, 82, 84, 86 and 88 or fragments comprising at least 50, 75, 100, 150, 200, 300 or more consecutive amino acids of one of the polypeptides of SEQ ID NOS: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 41, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 65, 67, 69, 70, 71, 74, 76, 78, 80, 82, 84, 86 and 88. The coding sequences of these nucleic acids may be identical to one of the coding sequences of one of the nucleic acids of SEQ ID NOS: 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 66, 68, 70, 72, 75, 77, 79, 81, 83, 85, 87 and 89 or a fragment thereof, or may be different coding sequences which encode one of the polypeptides of SEQ ID NOS: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 41, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 65, 67, 69, 70, 71, 74, 76, 78, 80, 82, 84, 86 and 88 or fragments comprising at least 50, 75, 100, 150, 200, 300 consecutive amino acids of one of the polypeptides of SEQ ID NOS: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 41, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 65, 67, 69, 70, 71, 74, 76, 78, 80, 82, 84, 86 and 88 as a result of the redundancy or degeneracy of the genetic code. The genetic code is well known to those of skill in the art and can be obtained, for example, from Stryer, *Biochemistry*, 3rd edition, W. H. Freeman & Co., New York.

The isolated, purified or enriched nucleic acid which encodes one of the polypeptides of SEQ ID NOS: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 41, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 65, 67, 69, 70, 71, 74, 76, 78, 80, 82, 84, 86 and 88 may include, but is not limited to: (1) only the coding sequences of one of SEQ ID NOS: 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 66, 68, 70, 72, 75, 77, 79, 81, 83, 85, 87 and 89; (2) the coding sequences of SEQ ID NOS: 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 66, 68, 70, 72, 75, 77, 79, 81, 83, 85, 87 and 89 and additional coding sequences, such as leader sequences or proprotein; and (3) the coding sequences of SEQ ID NOS: 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53,

55, 57, 59, 61, 63, 66, 68, 70, 72, 75, 77, 79, 81, 83, 85, 87 and 89 and non-coding sequences, such as non-coding sequences 5' and/or 3' of the coding sequence. Thus, as used herein, the term "polynucleotide encoding a polypeptide" encompasses a polynucleotide that includes only coding sequence for the polypeptide as well as a polynucleotide that includes additional coding and/or non-coding sequence.

The invention relates to polynucleotides based on SEQ ID NOS: 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 66, 68, 70, 72, 75, 77, 79, 81, 83, 85, 87 and 89 but having polynucleotide changes that are "silent", for example changes which do not alter the amino acid sequence encoded by the polynucleotides of SEQ ID NOS: 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 66, 68, 70, 72, 75, 77, 79, 81, 83, 85, 87 and 89. The invention also relates to polynucleotides which have nucleotide changes which result in amino acid substitutions, additions, deletions, fusions and truncations of the polypeptides of SEQ ID NOS: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 41, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 65, 67, 69, 70, 71, 74, 76, 78, 80, 82, 84, 86 and 88. Such nucleotide changes may be introduced using techniques such as site directed mutagenesis, random chemical mutagenesis, exonuclease III deletion, and other recombinant DNA techniques.

The isolated, purified or enriched nucleic acids of SEQ ID NOS: 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 66, 68, 70, 72, 75, 77, 79, 81, 83, 85, 87 and 89, the sequences complementary thereto, or a fragment comprising at least 100, 150, 200, 300, 400 or more consecutive bases of one of the sequence of SEQ ID NOS: 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 66, 68, 70, 72, 75, 77, 79, 81, 83, 85, 87 and 89, or the sequences complementary thereto may be used as probes to identify and isolate DNAs encoding the polypeptides of SEQ ID NOS: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 41, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 65, 67, 69, 70, 71, 74, 76, 78, 80, 82, 84, 86 and 88 espectively. In such procedures, a genomic DNA library is constructed from a

sample microorganism or a sample containing a microorganism capable of producing a farnesyl dibenzodiazepinone. The genomic DNA library is then contacted with a probe comprising a coding sequence or a fragment of the coding sequence, encoding one of the polypeptides of SEQ ID NOS: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 41, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 65, 67, 69, 70, 71, 74, 76, 78, 80, 82, 84, 86 and 88, or a fragment thereof under conditions which permit the probe to specifically hybridize to sequences complementary thereto. In a preferred embodiment, the probe is an oligonucleotide of about 10 to about 30 nucleotides in length designed based on a nucleic acid of SEQ ID NOS: 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 66, 68, 70, 72, 75, 77, 79, 81, 83, 85, 87 and 89. Genomic DNA clones which hybridize to the probe are then detected and isolated. Procedures for preparing and identifying DNA clones of interest are disclosed in Ausubel *et al.*, Current Protocols in Molecular Biology, John Wiley 503 Sons, Inc. 1997; and Sambrook *et al.*, Molecular Cloning: A Laboratory Manual 2d Ed., Cold Spring Harbor Laboratory Press, 1989. In another embodiment, the probe is a restriction fragment or a PCR amplified nucleic acid derived from SEQ ID NOS: 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37; 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 66, 68, 70, 72, 75, 77, 79, 81, 83, 85, 87 and 89.

The isolated, purified or enriched nucleic acids of SEQ ID NOS: 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 66, 68, 70, 72, 75, 77, 79, 81, 83, 85, 87 and 89, the sequences complementary thereto, or a fragment comprising at least 10, 15, 20, 25, 30, 35, 40, 50, 75, 100, 150, 200, 300, 400 or 500 consecutive bases of one of the sequences of SEQ ID NOS: 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 66, 68, 70, 72, 75, 77, 79, 81, 83, 85, 87 and 89 or the sequences complementary thereto may be used as probes to identify and isolate related nucleic acids. In some embodiments, the related nucleic acids may be genomic DNAs (or cDNAs) from potential farnesyl dibenzodiazepinone producers. In such procedures, a nucleic acid sample containing nucleic acids from a potential farnesyl

dibenzodiazepinone producer is contacted with the probe under conditions that permit the probe to specifically hybridize to related sequences. The nucleic acid sample may be a genomic DNA (or cDNA) library from the potential farnesyl dibenzodiazepinone-producer. Hybridization of the probe to nucleic acids is then detected using any of the methods described above.

Hybridization may be carried out under conditions of low stringency, moderate stringency or high stringency. As an example of nucleic acid hybridization, a polymer membrane containing immobilized denatured nucleic acids is first prehybridized for 30 minutes at 45 °C in a solution consisting of 0.9 M NaCl, 50 mM NaH_2PO_4, pH 7.0, 5.0 mM Na_2EDTA, 0.5% SDS, 10X Denhardt's, and 0.5 mg/ml polyriboadenylic acid. Approximately 2 x 10^7 cpm (specific activity 4-9 x 10^8 cpm/ug) of ^{32}P end-labeled oligonucleotide probe are then added to the solution. After 12-16 hours of incubation, the membrane is washed for 30 minutes at room temperature in 1X SET (150 mM NaCl, 20 mM Tris hydrochloride, pH 7.8, 1 mM Na_2EDTA) containing 0.5% SDS, followed by a 30 minute wash in fresh 1X SET at Tm-10°C for the oligonucleotide probe where Tm is the melting temperature. The membrane is then exposed to autoradiographic film for detection of hybridization signals.

By varying the stringency of the hybridization conditions used to identify nucleic acids, such as genomic DNAs or cDNAs, which hybridize to the detectable probe, nucleic acids having different levels of homology to the probe can be identified and isolated. Stringency may be varied by conducting the hybridization at varying temperatures below the melting temperatures of the probes. The melting temperature of the probe may be calculated using the following formulas:

For oligonucleotide probes between 14 and 70 nucleotides in length the melting temperature (Tm) in degrees Celcius may be calculated using the formula: Tm=81.5+16.6(log [Na+]) + 0.41(fraction G+C)-(600/N) where N is the length of the oligonucleotide.

If the hybridization is carried out in a solution containing formamide, the melting temperature may be calculated using the equation Tm=81.5+16.6(log [Na +]) +

0.41(fraction G + C)-(0.63% formamide)-(600/N) where N is the length of the probe.

Prehybridization may be carried out in 6X SSC, 5X Denhardt's reagent, 0.5% SDS, 0.1 mg/ml denatured fragmented salmon sperm DNA or 6X SSC, 5X Denhardt's reagent, 0.5% SDS, 0.1 mg/ml denatured fragmented salmon sperm DNA, 50% formamide. The composition of the SSC and Denhardt's solutions are listed in Sambrook et al., *supra*.

Hybridization is conducted by adding the detectable probe to the hybridization solutions listed above. Where the probe comprises double stranded DNA, it is denatured by incubating at elevated temperatures and quickly cooling before addition to the hybridization solution. It may also be desirable to similarly denature single stranded probes to eliminate or diminish formation of secondary structures or oligomerization. The filter is contacted with the hybridization solution for a sufficient period of time to allow the probe to hybridize to cDNAs or genomic DNAs containing sequences complementary thereto or homologous thereto. For probes over 200 nucleotides in length, the hybridization may be carried out at 15-25 °C below the Tm. For shorter probes, such as oligonucleotide probes, the hybridization may be conducted at 5-10 °C below the Tm. Preferably, the hybridization is conducted in 6X SSC, for shorter probes. Preferably, the hybridization is conducted in 50% formamide containing solutions, for longer probes. All the foregoing hybridizations would be considered to be examples of hybridization performed under conditions of high stringency.

Following hybridization, the filter is washed for at least 15 minutes in 2X SSC, 0.1% SDS at room temperature or higher, depending on the desired stringency. The filter is then washed with 0.1X SSC, 0.5% SDS at room temperature (again) for 30 minutes to 1 hour. Nucleic acids which have hybridized to the probe are identified by conventional autoradiography and non-radioactive detection methods.

The above procedure may be modified to identify nucleic acids having decreasing levels of homology to the probe sequence. For example, to obtain nucleic acids of decreasing homology to the detectable probe, less stringent conditions may be used. For example, the hybridization temperature may be decreased in increments of

5 °C from 68 °C to 42 °C in a hybridization buffer having a Na+ concentration of approximately 1M. Following hybridization, the filter may be washed with 2X SSC, 0.5% SDS at the temperature of hybridization. These conditions are considered to be "moderate stringency" conditions above 50°C and "low stringency" conditions below 50°C. A specific example of "moderate stringency" hybridization conditions is when the above hybridization is conducted at 55°C. A specific example of "low stringency" hybridization conditions is when the above hybridization is conducted at 45°C.

Alternatively, the hybridization may be carried out in buffers, such as 6X SSC, containing formamide at a temperature of 42 °C. In this case, the concentration of formamide in the hybridization buffer may be reduced in 5% increments from 50% to 0% to identify clones having decreasing levels of homology to the probe. Following hybridization, the filter may be washed with 6X SSC, 0.5% SDS at 50 °C. These conditions are considered to be "moderate stringency" conditions above 25% formamide and "low stringency" conditions below 25% formamide. A specific example of "moderate stringency" hybridization conditions is when the above hybridization is conducted at 30% formamide. A specific example of "low stringency" hybridization conditions is when the above hybridization is conducted at 10% formamide. Nucleic acids which have hybridized to the probe are identified by conventional autoradiography and non-radioactive detection methods.

The preceding methods may be used to isolate nucleic acids having at least 97%, at least 95%, at least 90%, at least 85%, at least 80%, or at least 70% sequence identity to a nucleic acid sequence selected from the group consisting of the sequences of SEQ ID NOS: 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 66, 68, 70, 72, 75, 77, 79, 81, 83, 85, 87 and 89. The isolated nucleic acid may have a coding sequence that is a naturally occurring allelic variant of one of the coding sequences described herein. Such allelic variant may have a substitution, deletion or addition of one or more nucleotides when compared to the nucleic acids of SEQ ID NOS: 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 66, 68, 70, 72,

75, 77, 79, 81, 83, 85, 87 and 89, or the sequences complementary thereto.

Additionally, the above procedures may be used to isolate nucleic acids which encode polypeptides having at least 99%, at least 95%, at least 90%, at least 85%, at least 80%, or at least 70% identity to a polypeptide having the sequence of one of SEQ ID NOS: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 41, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 65, 67, 69, 70, 71, 74, 76, 78, 80, 82, 84, 86 and 88 or fragments comprising at least 50, 75, 100, 150, 200, 300 consecutive amino acids thereof.

Another aspect of the present invention is an isolated or purified polypeptide comprising the sequence of one of SEQ ID NOS: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 41, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 65, 67, 69, 70, 71, 74, 76, 78, 80, 82, 84, 86 and 88 or fragments comprising at least 50, 75, 100, 150, 200 or 300 consecutive amino acids thereof. As discussed herein, such polypeptides may be obtained by inserting a nucleic acid encoding the polypeptide into a vector such that the coding sequence is operably linked to a sequence capable of driving the expression of the encoded polypeptide in a suitable host cell. For example, the expression vector may comprise a promoter, a ribosome binding site for translation initiation and a transcription terminator. The vector may also include appropriate sequences for modulating expression levels, an origin of replication and a selectable marker.

Promoters suitable for expressing the polypeptide or fragment thereof in bacteria include the *E.coli lac* or *trp* promoters, the lacI promoter, the lacZ promoter, the T3 promoter, the T7 promoter, the gpt promoter, the lambda P_R promoter, the lambda P_L promoter, promoters from operons encoding glycolytic enzymes such as 3-phosphoglycerate kinase (PGK), and the acid phosphatase promoter. Fungal promoters include the α factor promoter. Eukaryotic promoters include the CMV immediate early promoter, the HSV thymidine kinase promoter, heat shock promoters, the early and late SV40 promoter, LTRs from retroviruses, and the mouse metallothionein-I promoter. Other promoters known to control expression of genes in

prokaryotic or eukaryotic cells or their viruses may also be used.

Mammalian expression vectors may also comprise an origin of replication, any necessary ribosome binding sites, a polyadenylation site, splice donors and acceptor sites, transcriptional termination sequences, and 5' flanking nontranscribed sequences. In some embodiments, DNA sequences derived from the SV40 splice and polyadenylation sites may be used to provide the required nontranscribed genetic elements.

Vectors for expressing the polypeptide or fragment thereof in eukaryotic cells may also contain enhancers to increase expression levels. Enhancers are cis-acting elements of DNA, usually from about 10 to about 300 bp in length that act on a promoter to increase its transcription. Examples include the SV40 enhancer on the late side of the replication origin bp 100 to 270, the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and the adenovirus enhancers.

In addition, the expression vectors preferably contain one or more selectable marker genes to permit selection of host cells containing the vector. Examples of selectable markers that may be used include genes encoding dihydrofolate reductase or genes conferring neomycin resistance for eukaryotic cell culture, genes conferring tetracycline or ampicillin resistance in *E. coli*, and the *S. cerevisiae* TRP1 gene.

The appropriate DNA sequence may be inserted into the vector by a variety of procedures. In general, the DNA sequence is ligated to the desired position in the vector following digestion of the insert and the vector with appropriate restriction endonucleases. Alternatively, appropriate restriction enzyme sites can be engineered into a DNA sequence by PCR. A variety of cloning techniques are disclosed in Ausbel *et al.* Current Protocols in Molecular Biology, John Wiley 503 Sons, Inc. 1997 and Sambrook *et al.*, Molecular Cloning: A Laboratory Manual 2d Ed., Cold Spring Harbour Laboratory Press, 1989. Such procedures and others are deemed to be within the scope of those skilled in the art.

The vector may be, for example, in the form of a plasmid, a viral particle, or a phage. Other vectors include derivatives of chromosomal, nonchromosomal and

synthetic DNA sequences, viruses, bacterial plasmids, phage DNA, baculovirus, yeast plasmids, vectors derived from combinations of plasmids and phage DNA, viral DNA such as vaccinia, adenovirus, fowl pox virus, and pseudorabies. A variety of cloning and expression vectors for use with prokaryotic and eukaryotic hosts are described by Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor, N.Y., (1989).

Particular bacterial vectors which may be used include the commercially available plasmids comprising genetic elements of the well known cloning vector pBR322 (ATCC 37017), pKK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden), pGEM1 (Promega Biotec, Madison, WI, USA) pQE70, pQE60, pQE-9 (Qiagen), pD10, phiX174, pBluescript™ II KS, pNH8A, pNH16a, pNH18A, pNH46A (Stratagene), ptrc99a, pKK223-3, pKK233-3, pDR540, pRIT5 (Pharmacia), pKK232-8 and pCM7. Particular eukaryotic vectors include pSV2CAT, pOG44, pXT1, pSG (Stratagene) pSVK3, pBPV, pMSG, and pSVL (Pharmacia). However, any other vector may be used as long as it is replicable and stable in the host cell.

The host cell may be any of the host cells familiar to those skilled in the art, including prokaryotic cells or eukaryotic cells. As representative examples of appropriate hosts, there may be mentioned: bacteria cells, such as *E. coli*, *Streptomyces lividans*, *Streptomyces griseofuscus*, *Streptomyces ambofaciens*, *Bacillus subtilis*, *Salmonella typhimurium* and various species within the genera *Pseudomonas*, *Streptomyces*, *Bacillus,* and *Staphylococcus*, fungal cells, such as yeast, insect cells such as *Drosophila S2* and *Spodoptera Sf9*, animal cells such as CHO, COS or Bowes melanoma, and adenoviruses. The selection of an appropriate host is within the abilities of those skilled in the art.

The vector may be introduced into the host cells using any of a variety of techniques, including electroporation transformation, transfection, transduction, viral infection, gene guns, or Ti-mediated gene transfer. Where appropriate, the engineered host cells can be cultured in conventional nutrient media modified as appropriate for activating promoters, selecting transformants or amplifying the genes of the present invention. Following transformation of a suitable host strain and growth of the host

strain to an appropriate cell density, the selected promoter may be induced by appropriate means (e.g., temperature shift or chemical induction) and the cells may be cultured for an additional period to allow them to produce the desired polypeptide or fragment thereof.

Cells are typically harvested by centrifugation, disrupted by physical or chemical means, and the resulting crude extract is retained for further purification. Microbial cells employed for expression of proteins can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents. Such methods are well known to those skilled in the art. The expressed polypeptide or fragment thereof can be recovered and purified from recombinant cell cultures by methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. Protein refolding steps can be used, as necessary, in completing configuration of the polypeptide. If desired, high performance liquid chromatography (HPLC) can be employed for final purification steps.

Various mammalian cell culture systems can also be employed to express recombinant protein. Examples of mammalian expression systems include the COS-7 lines of monkey kidney fibroblasts (described by Gluzman, Cell, 23:175(1981)), and other cell lines capable of expressing proteins from a compatible vector, such as the C127, 3T3, CHO, HeLa and BHK cell lines. The constructs in host cells can be used in a conventional manner to produce the gene product encoded by the recombinant sequence. Polypeptides of the invention may or may not also include an initial methionine amino acid residue.

Alternatively, the polypeptides of SEQ ID NOS: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 41, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 65, 67, 69, 70, 71, 74, 76, 78, 80, 82, 84, 86 and 88 or fragments comprising at least 50, 75, 100, 150, 200 or 300 consecutive amino acids thereof can be synthetically produced by conventional peptide synthesizers. In other embodiments, fragments or portions of the

polynucleotides may be employed for producing the corresponding full-length polypeptide by peptide synthesis; therefore, the fragments may be employed as intermediates for producing the full-length polypeptides.

Cell-free translation systems can also be employed to produce one of the polypeptides of SEQ ID NOS: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 41, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 65, 67, 69, 70, 71, 74, 76, 78, 80, 82, 84, 86 and 88 or fragments comprising at least 50, 75, 100, 150, 200 or 300 consecutive amino acids thereof using mRNAs transcribed from a DNA construct comprising a promoter operably linked to a nucleic acid encoding the polypeptide or fragment thereof. In some embodiments, the DNA construct may be linearized prior to conducting an *in vitro* transcription reaction. The transcribed mRNA is then incubated with an appropriate cell-free translation extract, such as a rabbit reticulocyte extract, to produce the desired polypeptide or fragment thereof.

The present invention also relates to variants of the polypeptides of SEQ ID NOS: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 41, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 65, 67, 69, 70, 71, 74, 76, 78, 80, 82, 84, 86 and 88 or fragments comprising at least 50, 75, 100, 150, 200 or 300 consecutive amino acids thereof. The term "variant" includes derivatives or analogs of these polypeptides. In particular, the variants may differ in amino acid sequence from the polypeptides of SEQ ID NOS: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 41, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 65, 67, 69, 70, 71, 74, 76, 78, 80, 82, 84, 86 and 88 by one or more substitutions, additions, deletions, fusions and truncations, which may be present in any combination.

The variants may be naturally occurring or created *in vitro*. In particular, such variants may be created using genetic engineering techniques such as site directed mutagenesis, random chemical mutagenesis, exonuclease III deletion procedures, and standard cloning techniques. Alternatively, such variants, fragments, analogs, or derivatives may be created using chemical synthesis or modification procedures.

Other methods of making variants are also familiar to those skilled in the art. These include procedures in which nucleic acid sequences obtained from natural

isolates are modified to generate nucleic acids that encode polypeptides having characteristics which enhance their value in industrial or laboratory applications. In such procedures, a large number of variant sequences having one or more nucleotide differences with respect to the sequence obtained from the natural isolate are generated and characterized. Preferably, these nucleotide differences result in amino acid changes with respect to the polypeptides encoded by the nucleic acids from the natural isolates.

The variants of the polypeptides of SEQ ID NOS: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 41, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 65, 67, 69, 70, 71, 74, 76, 78, 80, 82, 84, 86 and 88 may be variants in which one or more of the amino acid residues of the polypeptides of SEQ ID NOS: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 41, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 65, 67, 69, 70, 71, 74, 76, 78, 80, 82, 84, 86 and 88 are substituted with a conserved or non-conserved amino acid residue (preferably a conserved amino acid residue) and such substituted amino acid residue may or may not be one encoded by the genetic code.

Conservative substitutions are those that substitute a given amino acid in a polypeptide by another amino acid of like characteristics. Typically seen as conservative substitutions are the following replacements: replacements of an aliphatic amino acid such as Ala, Val, Leu and Ile with another aliphatic amino acid; replacement of a Ser with a Thr or vice versa; replacement of an acidic residue such as Asp or Glu with another acidic residue; replacement of a residue bearing an amide group, such as Asn or Gln, with another residue bearing an amide group; exchange of a basic residue such as Lys or Arg with another basic residue; and replacement of an aromatic residue such as Phe or Tyr with another aromatic residue.

Other variants are those in which one or more of the amino acid residues of the polypeptides of SEQ ID NOS: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 41, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 65, 67, 69, 70, 71, 74, 76, 78, 80, 82, 84, 86 and 88 include a substituent group. Still other variants are those in which the polypeptide is associated with another compound, such as a compound to increase

the half-life of the polypeptide (for example, polyethylene glycol). Additional variants are those in which additional amino acids are fused to the polypeptide, such as leader sequence, a secretory sequence, a proprotein sequence or a sequence that facilitates purification, enrichment, or stabilization of the polypeptide.

In some embodiments, the fragments, derivatives and analogs retain the same biological function or activity as the polypeptides of SEQ ID NOS: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 41, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 65, 67, 69, 70, 71, 74, 76, 78, 80, 82, 84, 86 and 88. In other embodiments, the fragment, derivative or analogue includes a fused heterologous sequence that facilitates purification, enrichment, detection, stabilization or secretion of the polypeptide that can be enzymatically cleaved, in whole or in part, away from the fragment, derivative or analogue.

Another aspect of the present invention are polypeptides or fragments thereof which have at least 70%, at least 80%, at least 85%, at least 90%, or more than 95% identity to one of the polypeptides of SEQ ID NOS: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 41, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 65, 67, 69, 70, 71, 74, 76, 78, 80, 82, 84, 86 and 88 or a fragment comprising at least 50, 75, 100, 150, 200 or 300 consecutive amino acids thereof. It will be appreciated that amino acid "substantially identity" includes conservative substitutions such as those described above.

The polypeptides or fragments having homology to one of the polypeptides of SEQ ID NOS: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 41, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 65, 67, 69, 70, 71, 74, 76, 78, 80, 82, 84, 86 and 88 or a fragment comprising at least 50, 75, 100, 150, 200 or 300 consecutive amino acids thereof may be obtained by isolating the nucleic acids encoding them using the techniques described above.

Alternatively, the homologous polypeptides or fragments may be obtained through biochemical enrichment or purification procedures. The sequence of potentially homologous polypeptides or fragments may be determined by proteolytic digestion, gel electrophoresis and/or microsequencing. The sequence of the prospective homologous

polypeptide or fragment can be compared to one of the polypeptides of SEQ ID NOS: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 41, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 65, 67, 69, 70, 71, 74, 76, 78, 80, 82, 84, 86 and 88 or a fragment comprising at least 5, 10, 15, 20, 25, 30, 35, 40, 50, 75, 100, or 150 consecutive amino acids thereof.

The polypeptides of SEQ ID NOS: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 41, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 65, 67, 69, 70, 71, 74, 76, 78, 80, 82, 84, 86 and 88 or fragments, derivatives or analogs thereof comprising at least 40, 50, 75, 100, 150, 200 or 300 consecutive amino acids thereof invention may be used in a variety of applications. For example, the polypeptides or fragments, derivatives or analogs thereof may be used to catalyze biochemical reactions as described elsewhere in the specification.

VI. Pharmaceutical compositions comprising farnesyl dibenzodiazepinones

In another embodiment, the invention relates to a pharmaceutical composition comprising a farnesyl dibenzodiazepinone, as described in the preceding section, and a pharmaceutically acceptable carrier, as described below. The pharmaceutical composition comprising the farnesyl dibenzodiazepinone is useful for treating a variety of diseases and disorders, including cancer, inflammation and bacterial infections.

The compounds of the present invention, or pharmaceutically acceptable salts thereof, can be formulated for oral, intravenous, intramuscular, subcutaneous, topical or parenteral administration for the therapeutic or prophylactic treatment of diseases, particularly bacterial infections, acute and chronic inflammation and cancer. For oral or parental administration, compounds of the present invention can be mixed with conventional pharmaceutical carriers and excipients and used in the form of tablets, capsules, elixirs, suspensions, syrups, wafers and the like. The compositions comprising a compound of this present invention will contain from about 0.1% to about 99.9%, about 1% to about 98%, about 5% to about 95%, about 10% to about 80% or about 15% to about 60% by weight of the active compound.

The pharmaceutical preparations disclosed herein are prepared in accordance

with standard procedures and are administered at dosages that are selected to reduce, prevent, or eliminate bacterial infection, cancer or inflammation. (See, e.g., *Remington's Pharmaceutical Sciences*, Mack Publishing Company, Easton, PA; and Goodman and Gilman, *Pharmaceutical Basis of Therapeutics*, Pergamon Press, New York, NY, the contents of which are incorporated herein by reference, for a general description of the methods for administering various antimicrobial agents for human therapy). The compositions of the present invention can be delivered using controlled (e.g., capsules) or sustained release delivery systems (e.g., bioerodable matrices). Exemplary delayed release delivery systems for drug delivery that are suitable for administration of the compositions of the invention (preferably of Formula I) are described in U.S. Patent Nos 4,452,775 (issued to Kent), 5,239,660 (issued to Leonard), 3,854,480 (issued to Zaffaroni).

The pharmaceutically acceptable compositions of the present invention comprise one or more compounds of the present invention in association with one or more non-toxic, pharmaceutically acceptable carriers and/or diluents and/or adjuvants and/or excipients, collectively referred to herein as "carrier" materials, and if desired other active ingredients. The compositions may contain common carriers and excipients, such as corn starch or gelatin, lactose, sucrose, microcrystalline cellulose, kaolin, mannitol, dicalcium phosphate, sodium chloride and alginic acid. The compositions may contain crosarmellose sodium, microcrystalline cellulose, sodium starch glycolate and alginic acid.

Tablet binders that can be included are acacia, methylcellulose, sodium carboxymethylcellulose, polyvinylpyrrolidone (Providone), hydroxypropyl methylcellulose, sucrose, starch and ethylcellulose.

Lubricants that can be used include magnesium stearate or other metallic stearates, stearic acid, silicon fluid, talc, waxes, oils and colloidal silica.

Flavouring agents such as peppermint, oil of wintergreen, cherry flavouring or the like can also be used. It may also be desirable to add a coloring agent to make the dosage form more aesthetic in appearance or to help identify the product comprising a compound of the present invention.

For oral use, solid formulations such as tablets and capsules are particularly useful. Sustained released or enterically coated preparations may also be devised. For pediatric and geriatric applications, suspension, syrups and chewable tablets are especially suitable. For oral administration, the pharmaceutical compositions are in the form of, for example, a tablet, capsule, suspension or liquid. The pharmaceutical composition is preferably made in the form of a dosage unit containing a therapeutically-effective amount of the active ingredient. Examples of such dosage units are tablets and capsules. For therapeutic purposes, the tablets and capsules which can contain, in addition to the active ingredient, conventional carriers such as binding agents, for example, acacia gum, gelatin, polyvinylpyrrolidone, sorbitol, or tragacanth; fillers, for example, calcium phosphate, glycine, lactose, maize-starch, sorbitol, or sucrose; lubricants, for example, magnesium stearate, polyethylene glycol, silica or talc: disintegrants, for example, potato starch, flavoring or coloring agents, or acceptable wetting agents. Oral liquid preparations generally are in the form of aqueous or oily solutions, suspensions, emulsions, syrups or elixirs and may contain conventional additives such as suspending agents, emulsifying agents, non-aqueous agents, preservatives, coloring agents and flavoring agents. Examples of additives for liquid preparations include acacia, almond oil, ethyl alcohol, fractionated coconut oil, gelatin, glucose syrup, glycerin, hydrogenated edible fats, lecithin, methyl cellulose, methyl or propyl para-hydroxybenzoate, propylene glycol, sorbitol, or sorbic acid.

For intravenous (iv) use, compounds of the present invention can be dissolved or suspended in any of the commonly used intravenous fluids and administered by infusion. Intravenous fluids include, without limitation, physiological saline or Ringer's solution.

Formulations for parental administration can be in the form of aqueous or non-aqueous isotonic sterile injection solutions or suspensions. These solutions or suspensions can be prepared from sterile powders or granules having one or more of the carriers mentioned for use in the formulations for oral administration. The compounds can be dissolved in polyethylene glycol, propylene glycol, ethanol, corn oil, benzyl alcohol, sodium chloride, and/or various buffers.

For intramuscular preparations, a sterile formulation of compounds of the present invention or suitable soluble salts forming the compound, can be dissolved and administered in a pharmaceutical diluent such as Water-for-Injection (WFI), physiological saline or 5% glucose. A suitable insoluble form of the compound may be prepared and administered as a suspension in an aqueous base or a pharmaceutically acceptable oil base, e.g. an ester of a long chain fatty acid such as ethyl oleate.

For topical use the compounds of present invention can also be prepared in suitable forms to be applied to the skin, or mucus membranes of the nose and throat, and can take the form of creams, ointments, liquid sprays or inhalants, lozenges, or throat paints. Such topical formulations further can include chemical compounds such as dimethylsulfoxide (DMSO) to facilitate surface penetration of the active ingredient.

For application to the eyes or ears, the compounds of the present invention can be presented in liquid or semi-liquid form formulated in hydrophobic or hydrophilic bases as ointments, creams, lotions, paints or powders.

For rectal administration the compounds of the present invention can be administered in the form of suppositories admixed with conventional carriers such as cocoa butter, wax or other glyceride.

Alternatively, the compound of the present invention can be in powder form for reconstitution in the appropriate pharmaceutically acceptable carrier at the time of delivery. In another embodiment, the unit dosage form of the compound can be a solution of the compound or a salt thereof in a suitable diluent in sterile, hermetically sealed ampoules.

The amount of the compound of the present invention in a unit dosage comprises a therapeutically-effective amount of at least one active compound of the present invention which may vary depending on the recipient subject, route and frequency of administration. A recipient subject refers to a plant, a cell culture or an animal such as an ovine or a mammal including a human.

According to this aspect of the present invention, the novel compositions disclosed herein are placed in a pharmaceutically acceptable carrier and are delivered to a recipient subject (including a human subject) in accordance with known methods of

drug delivery. In general, the methods of the invention for delivering the compositions of the invention in vivo utilize art-recognized protocols for delivering the agent with the only substantial procedural modification being the substitution of the compounds of the present invention for the drugs in the art-recognized protocols.

Likewise, the methods for using the claimed composition for treating cells in culture, for example, to eliminate or reduce the level of bacterial contamination of a cell culture, utilize art-recognized protocols for treating cell cultures with antibacterial agent(s) with the only substantial procedural modification being the substitution of the compounds of the present invention for the agents used in the art-recognized protocols.

The compounds of the present invention provide a method for treating bacterial infections, pre-cancerous or cancerous conditions, and acute or chronic inflammatory disease. As used herein, the term "unit dosage" refers to a quantity of a therapeutically effective amount of a compound of the present invention that elicits a desired therapeutic response. As used herein, the phrase "therapeutically effective amount" means an amount of a compound of the present invention that prevents the onset, alleviates the symptoms, or stops the progression of a bacterial infection, inflammatory condition, or pre-cancerous or cancerous condition. The term "treating" is defined as administering, to a subject, a therapeutically effective amount of at least one compound of the present invention, both to prevent the occurrence of a bacterial infection, inflammation or pre-cancer or cancer condition, or to control or eliminate a bacterial infection, inflammation or pre-cancer or cancer condition. The term "desired therapeutic response" refers to treating a recipient subject with a compound of the present invention such that a bacterial or inflammatory condition or pre-cancer or cancer condition is reversed, arrested or prevented in a recipient subject.

The compounds of the present invention can be administered as a single daily dose or in multiple doses per day. The treatment regime may require administration over extended periods of time, e.g., for several days or for from two to four weeks. The amount per administered dose or the total amount administered will depend on such factors as the nature and severity of the disease condition, the age and general health of the recipient subject, the tolerance of the recipient subject to the compound and the

type of the bacterial infection, inflammatory disorder, or type of cancer.

A compound according to this invention may also be administered in the diet or feed of a patient or animal. The diet for animals can be normal foodstuffs to which the compound can be added or it can be added to a premix.

The compounds of the present invention may be taken in combination, together or separately with any known clinically approved antibiotic, inflammation or anti-cancer agent to treat a recipient subject in need of such treatment.

VII. Method of Inhibiting Tumor Growth

In another embodiment, the present invention relates to a method of inhibiting tumor growth. Compounds as described herein can possess antitumor activity. The compounds are effective against mammalian tumor cells such as leukemia cells, melanoma cells, breast carcinoma cells, lung carcinoma cells, pancreatic carcinoma cells, ovarian carcinoma cells, renal carcinoma cells, colon carcinoma cells prostate carcinoma cells and glioma cells. The antitumor method of the invention results in inhibition of tumor cells. The term "inhibition", when used in conjunction with the antitumor method refers to suppression, killing, stasis, or destruction of tumor cells. The antitumor method preferably results in prevention, reduction or elimination of invasive activity and related metastasis of tumor cells. The term "effective amount" when used in conjunction with the antitumor cell method refers to the amount of the compound sufficient to result in the inhibition of mammalian tumor cells.

The inhibition of mammalian tumor growth according to this method can be monitored in several ways. First, tumor cells grown in vitro can be treated with the compound and monitored for growth or death relative to the same cells cultured in the absence of the compound. A cessation of growth or a slowing of the growth rate (i.e., the doubling rate), e.g., by 10% or more, is indicative of tumor cell inhibition. Alternatively, tumor cell inhibition can be monitored by administering the compound to an animal model of the tumor of interest. Examples of experimental animal tumor models are known in the art and described in the examples herein. A cessation of tumor growth (i.e., no further increase in size) or a reduction in tumor size (i.e., tumor

volume) or cell number (e.g., at least a 10% decrease in either) in animals treated with a compound as described herein relative to tumors in control animals not treated with the compound is indicative of tumor growth inhibition.

To monitor the efficacy of tumor treatment in a human, tumor size or tumor cell titer is measured before and after initiation of the treatment, and treatment is considered effective if either the tumor size or titer ceases further growth, or if the tumor is reduced in size or titer, e.g., by at least 10% or more (e.g., 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or even 100%, that is, the absence of the tumor). Methods of determining the size or cell titer of a tumor in vivo vary with the type of tumor, and include, for example, various imaging techniques well known to those in the medical imaging or oncology fields (MRI, CAT, PET, etc.), as well as histological techniques and flow cytometry.

For the antitumor method of the invention, a typical effective dose of the compounds given orally or parenterally would be from about 5 to about 100 mg/kg of body weight of the subject with a daily dose ranging from about 15 to about 300 mg/kg of body weight of the subject.

VIII. Method of Inhibiting Lipoxygenase

In another embodiment, the present invention also provides for a method of treating diseased states, in particular inflammation, caused by the 5-lipoxygenase system and/or by the synthesis of the Leukotrienes C_4, D_4, E_4 and F_4 as well as Leukotriene B_4 in mammals, especially in human subjects. This method comprises administering to a subject an effective amount of ECO-04601. Compound ECO-04601 may be used alone or in combination with other anti-inflammatory compounds to treat or prevent disease states related to inflammation including pulmonary conditions, inflammation, cardiovascular conditions, central nervous system conditions or skin conditions. More specific diseases include gastritis; erosive esophagitis; inflammatory bowel disease; ethanol-induced hemorrhagic erosions; hepatic ischemia; ischemic neuronal injury; noxious agent induced damage or necrosis of hepatic, pancreatic, renal, neuronal or myocardial tissue; liver parenchymal damage caused by hepatoxic

agents such as CCl_4 and D-galactosamine; ischemic renal failure; disease-induced hepatic damage; trauma- or stress-induced cell damage; asthma; multiple sclerosis; ischemic reperfusion; edema; rheumatoid arthritis; viral encephalitis; bacterial pneumonia; neurodegeneration; Alzheimer's disease and glycerol-induced renal failure.

For the method of the invention related to the 5-lipoxygenase system and/or the biosynthesis of Leukotrienes, a typical effective unit dose of ECO-04601 given orally or parenterally would be from about 5 to about 100 mg/kg of body weight of the subject with a daily dose ranging from about 15 to about 300 mg/kg of body weight of the subject.

The inhibition of lipoxygenase enzymes is monitored using methods well known in the art and as described in the examples herein. A decrease in enzyme activity by at least 10%, relative to the activity in the absence of a compound as described herein is indicative of effective inhibition of lipoxygenase activity.

Farnesyl dibenzodiazepinone compounds useful according to the invention can be used to reduce or prevent inflammation. Among the hallmarks of local acute inflammation are heat, redness, swelling, pain and loss of function. These changes are induced largely by changes in vascular flow and caliber, changes in vascular permeability and leukocyte exudation (Robbins et al., "Pathologic Basis of Disease", 6th Ed., W.B. Saunders Co., Philadelphia, PA). Anti-inflammatory therapy performed using compounds useful according to the invention can be monitored for success by tracking any of these changes. For example, a decrease in swelling (e.g., at least 10% decrease following treatment) or reported pain (e.g., a sustained decrease of 1 point or more on a 1-10 scale reported by the patient, with 10 being the worst pain experienced in association with this disorder prior to treatment, and 0 being no pain) can be used to indicate successful treatment.

Other measurable hallmarks of inflammation include leukocyte infiltration and inflammatory cytokine levels. These hallmarks can be monitored by biopsy of the affected tissue. A decrease of 10% or more in leukocyte infiltration in fixed, stained tissue relative to infiltration in similar tissue prior to treatment can be used to indicate successful treatment, as can a decrease of 10% or more in the level of any given

inflammatory cytokine, relative to the level before treatment. Those skilled in the art can readily assay for inflammatory cytokine levels in tissue, blood, or other fluid samples. Alternatively, the level of systemic indicators of inflammation such as C reactive protein levels and erythrocyte sedimentation rate can be monitored. Each of these has established normal ranges in medicine, and treatment is considered successful if one or more of such indicators goes from outside the normal range to inside the normal range after the initiation of treatment.

IX. Method of Inhibiting Bacterial Growth

In another embodiment, the present invention relates to a method for treating bacterial infection in a mammalian subject in need thereof, comprising the step of administering to the mammal a therapeutically effective amount of compound ECO-04601, a compound as described herein, or a pharmaceutically acceptable derivative or prodrug thereof.

According to another embodiment, the invention provides a method of decreasing bacterial quantity in a biological sample. This method comprises the step of contacting the biological sample with a compound ECO-04601, a compound as described herein, or a pharmaceutically acceptable derivative or prodrug thereof. This method is effective if the number of bacteria decreases by at least 10%, and preferably more, e.g., 25%, 50%, 75% or even 100% after contacting the biological sample with compound ECO-04601, a compound as described herein, or a pharmaceutically acceptable derivative or prodrug thereof.

These pharmaceutical compositions effective to treat or prevent a bacterial infection which comprise ECO-04601, a compound as described herein, or a pharmaceutically acceptable derivative or prodrug thereof in an amount sufficient to measurably decrease bacterial quantity, and a pharmaceutically acceptable carrier, are another embodiment of the present invention. The term "measurably decrease bacterial quantity", as used herein means a measurable change in the number of bacteria between a sample containing the inhibitor and a sample not containing the inhibitor.

Agents which increase the susceptibility of bacterial organisms to antibiotics are

known. For example, U.S. Pat. No. 5,523,288, U.S. Pat. No. 5,783,561 and U.S. Pat. No. 6,140,306 describe methods of using bactericidal/permeability-increasing protein (BPI) for increasing antibiotic susceptibility of gram-positive and gram-negative bacteria. Agents that increase the permeability of the outer membrane of bacterial organisms have been described by Vaara, M. in Microbiological Reviews (1992) pp. 395-411, and the sensitization of gram-negative bacteria has been described by Tsubery, H., et al, in J. Med. Chem . (2000) pp. 3085-3092.

For the method of the invention related to treatment of subjects with a bacterial infection, a typical effective unit dose of ECO-04601, a compound described herein or a pharmaceutically acceptable derivative or prodrug thereof given orally or parenterally would be from about 5 to about 100 mg/kg of body weight of the subject with a daily dose ranging from about 15 to about 300 mg/kg of body weight of the subject.

Another preferred embodiment of this invention relates to a method, as described above, of treating a bacterial infection in a mammal in need thereof, but further comprising the step of administering to the mammal an agent which increases the susceptibility of bacterial organisms to antibiotics.

According to another preferred embodiment, the invention provides a method, as described above, of decreasing bacterial quantity in a biological sample, but further comprising the step of contacting the biological sample with an agent which increases the susceptibility of bacterial organisms to antibiotics.

Methods of decreasing bacterial quantity are effective if the number of bacteria decreases at least 10%, and preferably more, e.g., 25%, 50%, 75% or even 100% after contacting the biological sample with compound ECO-04601, a compound as described herein, or a pharmaceutically acceptable derivative or prodrug thereof.

The pharmaceutical compositions and methods of this invention will be useful generally for controlling bacterial infections *in vivo*. Examples of bacterial organisms that may be controlled by the compositions and methods of this invention include, but are not limited to the following organisms: *Streptococcus pneumoniae, Streptococcus pyrogenes, Enterococcus fecalis, Enterococcus faecium, Klebsiella pneumoniae, Enterobacter spp., Proteus spp., Pseudomonas aeruginosa, E. coli, Serratia*

marcesens, Staphylococcus aureus, Coagulase negative *Staphylococcus, Haemophilus infuenzae, Bacillus anthracis, Mycoplasma pneumoniae*, and *Staphylococcus epidermidis*. The compositions and methods will therefore be useful for controlling, treating or reducing the advancement, severity or effects of nosocomial or non-nosocomial infections. Examples of nosocomial uses include, but are not limited to, urinary tract infections, pneumonia, surgical wound infections, bacteremia and therapy for febrile neutropenic patients. Examples of non-nosocomial uses include but are not limited to urinary tract infections, pneumonia, prostatitis, skin and soft tissue infections and intra-abdominal infections.

In addition to the compounds of this invention, pharmaceutically acceptable derivatives or prodrugs of the compounds of this invention may also be employed in compositions to treat or prevent the above-identified disorders.

A "pharmaceutically acceptable derivative or prodrug" means any pharmaceutically acceptable salt, ester, salt of an ester or other derivative of a compound of this invention which, upon administration to a recipient, is capable of providing, either directly or indirectly, a compound of this invention or an inhibitorily active metabolite or residue thereof. Particularly favored derivatives or prodrugs are those that increase the bioavailability of the compounds of this invention when such compounds are administered to a mammal (e.g., by allowing an orally administered compound to be more readily absorbed into the blood) or which enhance delivery of the parent compound to a biological compartment (e.g., the brain or lymphatic system) relative to the parent species.

Pharmaceutically acceptable prodrugs of the compounds of this invention include, without limitation, esters, amino acid esters, phosphate esters, metal salts and sulfonate esters.

Unless otherwise indicated, all numbers expressing quantities of ingredients, properties such as molecular weight, reaction conditions, IC_{50} and so forth used in the specification and claims are to be understood as being modified in all instances by the term “about”. Accordingly, unless indicated to the contrary, the numerical parameters set forth in the present specification and attached claims are approximations. At the

very least, and not as an attempt to limit the application of the doctrine of equivalents to the scope of the claims, each numerical parameter should at least be construed in light of the number of significant figures and by applying ordinary rounding techniques. Notwithstanding that the numerical ranges and parameters setting forth the broad scope of the invention are approximations, the numerical values set in the examples, Tables and Figures are reported as precisely as possible. Any numerical values may inherently contain certain errors resulting from variations in experiments, testing measurements, statistical analyses and such.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

EXAMPLES

EXAMPLE 1: PREPARATION OF PRODUCTION CULTURE

Unless otherwise noted, all reagents were purchased from Sigma Chemical Co. (St. Louis, MO), (Aldrich). *Micromonospora spp.* (deposit accession number IDAC 070303-01) was maintained on agar plates of ISP2 agar (Difco Laboratories, Detroit, MI). An inoculum for the production phase was prepared by transferring the surface growth of the *Micromonospora spp.* from the agar plates to 125-mL flasks containing 25 mL of sterile medium comprised of 24 g potato dextrin, 3 g beef extract, 5 g Bacto-casitone, 5 g glucose, 5 g yeast extract, and 4 g $CaCO_3$ made up to one liter with distilled water (pH 7.0). The culture was incubated at about 28°C for approximately 60

hours on a rotary shaker set at 250 rpm. Following incubation, 10 mL of culture was transferred to a 2L baffled flask containing 500 mL of sterile production medium containing 20 g/L potato dextrin, 20 g/L glycerol, 10 g/L Fish meal, 5 g/L Bacto-peptone, 2 g/L $CaCO_3$, and 2 g/L $(NH_4)_2SO_4$, pH 7.0. Fermentation broth was prepared by incubating the production culture at 28°C in a rotary shaker set at 250 rpm for one week.

EXAMPLE 2: ISOLATION

500 mL ethyl acetate was added to 500 mL of fermentation broth prepared as described in Example 1 above. The mixture was agitated for 30 minutes on an orbital shaker at 200 rpm to create an emulsion. The phases were separated by centrifugation and decantation. Between 4 and 5 g of anhydrous $MgSO_4$ was added to the organic phase, which was then filtered and the solvents removed in vacuo.

An ethyl acetate extract from 2 L fermentation was mixed with HP-20 resin (100 mL; Mitsubishi Casei Corp., Tokyo, Japan) in water (300 mL). Ethyl acetate was removed in vacuo, the resin was filtered on a Buchner funnel and the filtrate was discarded. The adsorbed HP-20 resin was then washed successively with 2 × 125 mL of 50% acetonitrile in water, 2×125 mL of 75% acetonitrile in water and 2 × 125 mL of acetonitrile.

Fractions containing the compound of Formula II were evaporated to dryness and 100 mg was digested in the 5 mL of the upper phase of a mixture prepared from chloroform, cyclohexane, methanol, and water in the ratios, by volume, of 5:2:10:5. The sample was subjected to centrifugal partition chromatography using a High Speed Countercurrent (HSCC) system (Kromaton Technologies, Angers, France) fitted with a 200 mL cartridge and prepacked with the upper phase of this two-phase system. The HSCC was run with the lower phase mobile and the compound of Formula II was eluted at approximately one-half column volume. Fractions were collected and the compound of Formula II was detected by TLC of aliquots of the fractions on commercial Kieselgel

$60F_{254}$ plates. Compound could be visualized by inspection of dried plates under UV light or by spraying the plates with a spray containing vanillin (0.75%) and concentrated sulfuric acid (1.5%, v/v) in ethanol and subsequently heating the plate. Fractions contained substantially pure compound of Formula II, although highly colored. A buff-colored sample could be obtained by chromatography on HPLC as follows.

6 mg of sample was dissolved in acetonitrile and injected onto a preparative HPLC column (XTerra ODS (10μm), 19×150mm, Waters Co., Milford, MA), with a 9 mL/min flow rate and UV peak detection at 300 nm. The column was eluted with acetonitrile/buffer (20 mM of NH_4HCO_3) according to the following gradient shown in Table 1

Table 1

Time (min)	Water (%)	Acetonitrile (%)
0	70	30
10	5	95
15	5	95
20	70	30

Fractions containing the compound of Formula II eluted at approximately 11:0 min and were combined, concentrated and lyophilized to give a yield of 3.8 mg compound.

Alternative Protocol 1

The compound of Formula II was also isolated using the following alternative protocol. At the end of the incubation period, the fermentation broth from the baffled flasks of Example 1 was centrifuged and the supernatant decanted from the pellet containing the bacterial mycelia. 100 mL of 100% MeOH was added to the mycelial pellet and the sample was stirred for 10 minutes and centrifuged for 15 minutes. The methanolic supernatant was decanted and saved. 100 mL of acetone was then added to the mycelial pellet and stirred for 10 minutes then centrifuged for 15 minutes. The

acetonic supernatant was decanted and combined with the methanolic supernatant. Finally, 100 mL of 20% MeOH/H_2O was added to the mycelial pellet, stirred for 10 minutes and centrifuged for 15 minutes. The supernatant was combined with the acetonic and methanolic supernatants.

The combined supernatant was added to 400 ml of HP-20 resin in 1000 mL of water and the organics were removed *in vacuo*. The resulting slurry was filtered on a Buchner funnel and the filtrate was discarded. Adsorbed HP-20 resin was washed successively with 2×500mL of 50% MeOH/H_2O, 2×500mL of 75% MeOH/H_2O and 2×500mL of MeOH.

The individual washes were collected separately and analyzed by TLC as described above. Those fractions containing the compound of Formula II were evaporated to near dryness and lyophilized. The lyophilizate was dissolved in methanol and injected onto a preparative HPLC column (Xterra ODS (10μm), 19×150mm, Waters Co., Milford, MA) with a flow rate of 9 mL/min and peak detection at 300 nm.

The column was eluted with acetonitrile/buffer (5 mM of NH_4HCO_3) according to gradient shown in Table 2.

Table 2

Time (min)	Buffer (%)	Acetonitrile (%)
0	95	5
15	45	55
20	5	95
30	5	95
35	95	5

Fractions containing the compound of Formula II were combined, concentrated and lyophilized to yield about 33.7 mg of compound.

Alternative Protocol 2

10 liters of the whole broth from Example 1 are extracted twice with equal volumes of ethyl acetate and the two extracts are combined and concentrated to dryness. The dried extract is weighed, and for every gram of dry extract, 100 mL of MeOH-H_2O (2:1 v/v) and 100 mL of hexane is added. The mixture is swirled gently but

well to achieve dissolution. The two layers are separated and the aqueous layer is washed with 100 mL of hexane. The two hexane layers are combined and the combined hexane solution is washed with 100 mL methanol:water (2:1, v/v). The two methanol:water layers are combined and treated with 200 mL of EtOAc and 400 mL of water. The layers are separated and the aqueous layer is extracted twice more with 200 mL portions of EtOAc. The EtOAc layers are combined and concentrated. The residue obtained will be suitable for final purification, either by HSCC or by HPLC as described above. This extraction process achieves a ten-fold purification when compared with the extraction protocol used above.

EXAMPLE 3: ELUCIDATION OF THE STRUCTURE OF COMPOUND OF FORMULA II.

The structure of the compound of Formula II was derived from spectroscopic data, including mass, UV, and NMR spectroscopy. Mass was determined by electrospray mass spectrometry to be 462.6 (FIGURE 1), UVmax 230nm with a shoulder at 290 nm (FIGURE 2). NMR data were collected dissolved in MeOH-*d4* including proton (FIGURE 3), and multidimensional pulse sequences gDQCOSY (FIGURE 4), gHSQC (FIGURE 5), gHMBC (FIGURE 6), and NOESY (FIGURE 7).

A number of cross peaks in the 2D spectra of ECO-04601 are key in the structural determination. For example, the farnesyl chain is placed on the amide nitrogen by a strong cross peak between the proton signal of the terminal methylene of that chain at 4.52 ppm and the amide carbonyl carbon at 170 ppm in the gHMBC experiment. This conclusion is confirmed by a cross peak in the NOESY spectrum between the same methylene signals at 4.52 ppm and the aromatic proton signal at 6.25 ppm from one of the two protons of the tetra substituted benzenoid ring.

Based on the mass, UV and NMR spectroscopy data, the structure of the compound was determined to be the structure of Formula II.

EXAMPLE 4: ANTIBACTERIAL ACTIVITY (MINIMAL INHIBITORY

CONCENTRATION DETERMINATION)

Minimal Inhibitory Concentration (MIC) is defined as the lowest concentration of drug that inhibits more than 99% of the bacterial population. The MIC determination of ECO-04601 against bacteria strains (*Bacillus subtilis* – ATCC 23857; *Micrococcus luteus* – ATCC 9341) was performed using broth microdilution assay (Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard-Fifth Edition. NCCLS document M7-A5 (ISBN 1-56238-394-9). NCCLS, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898 USA.).

Test compound preparation: The test article ECO-04601 is prepared as 100X stock solutions in DMSO, with concentrations ranging from 3.2 mg/ml to 0.0625 mg/ml (a two-fold dilution series over 10 points). The first dilution (3.2mg/ml) was prepared by resuspending 0.5 mg of each test article in 156.25 µl of DMSO. The stock is then serially diluted by two-fold decrement to obtain the desired concentration range.

Inoculum preparation: From an overnight culture in Mueller Hinton (MH) broth, cell density for each indicator strain (*Bacillus subtilis*; *Micrococcus luteus*) was adjusted to 0.5 Mc Farland units in 0.85% saline, then further diluted 1/100 in appropriate assay medium (~ 1 X 10^6 cells/ml).

MIC determination: The 100X ECO-04601 solutions was diluted 50 times in MH broth and dispensed in a 96 well plate, one test concentration per column of wells, 10 columns in total. The 11^{th} column of wells contained MH broth with 1% DMSO, the 12^{th} column of wells contained 100 µl of broth alone. 50 µl of the final cell dilution of each indicator strain was added to each corresponding well of the microplate containing 50 µl of diluted drug or media alone. Assay plates were incubated at 35°C for 24 hrs.

The results of the MIC for the compound of ECO-04601, shown in Table 3, demonstrate a range of antibacterial effects:

Table 3

Indicator strain	MIC (µg/mL)
Bacillus subtilis ATCC 23857	12.5
Micrococcus luteus ATCC 9341	6.25

EXAMPLE 5. ANTICANCER ACTIVITY *IN VITRO* AGAINST HUMAN AND ANIMAL TUMOR CELL LINES FROM VARIOUS TISSUES

Culture conditions: The cell lines listed in Table 4 were used to characterize the cytotoxicity of ECO-04601 against human and animal tumor cell lines. These cell lines were shown to be free of mycoplasma infection and were maintained on the appropriate media (Table 4) supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin-streptomycin, under 5% CO_2 at 37°C. Cells were passaged twice to three times per week. Viability was examined by staining with 0.25% trypan blue and only flasks where cell viability was >95% were used for this study.

Cell lines amplification and plating: Tumor cells were seeded (1-3 x 10^3 cells per 100 µL) in 96-wells flat bottom microtiter plates and incubated at 37°C and 5% CO_2 for 16 hrs before treatment in drug-free medium supplemented with 10% serum.

Evaluation of inhibitory activity on cell proliferation: Cells were incubated for 96 hrs with 6 $\log_{10}$-fold concentrations of the test substance starting at 10µg/ml (20 µM). The test substance stock solution (5 mg/mL) was initially diluted at 1/70 fold in medium supplemented with serum. Other concentrations were then obtained from 1/10 fold successive dilutions in the same supplemented medium. Cell survival was evaluated 96 h later by replacing the culture media with 150 µL fresh medium containing 10 mM 4-(2-

hydroxyethyl)-1-piperazineethanesulfonic acid buffer, pH 7.4. Next, 50 µL of 2.5 mg/mL of 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in phosphate buffer solution, pH 7.4, was added. After 3-4h of incubation at 37°C, the medium and soluble MTT was removed, and 200 µL of dimethylsulfoxide was added to dissolve the precipitate of reduced MTT followed by addition of 25 µL glycine buffer (0.1 M glycine plus 0.1 M NaCl, pH 10.5). The absorbance was determined at 570 nm with a microplate reader. Results were expressed as the concentration of drug which inhibits 50% of the cell growth (IC_{50}). The IC_{50} values shown in Table 4 demonstrated a pharmacologically relevant cytotoxic activity of ECO-04601 against a variety of tumor types such as leukemias, melanomas, pancreatic and breast carcinomas.

Table 4

Cell lines	Type	Origin	Source	Culture medium	IC_{50} ($x10^{-6}M$)
K562	Leukemia myelogeneous	Human	ATCC	RPMI 1640	8.6
P388	Leukemia	Mouse	ATCC	RPMI 1640	10.9
I83	Leukemia	Human	ATCC	RPMI 1640	2.7
B16 (F10)	Melanoma	Mouse	ATCC	RPMI 1640	11.4
SK-MEL 28	Melanoma	Human	ATCC	RPMI 1640	14.0
SK-MEL 28^{VEGF}	Melanoma (expressing VEGF)	Human	ATCC	RPMI 1640	14.3
SK-MEL-1	Melanoma	Human	ATCC	EMEM 1% non-essential amino acid 1% Sodium puryvate	14.1
Panc 96	Pancreatic carcinoma	Human	ATCC	RPMI 1% Sodium puryvate	12.5
Panc 10.05	Pancreatic carcinoma	Human	ATCC	RPMI 1% Sodium puryvate Insulin	14.2
MCF-7	Breast adenocarcinoma	Human	ATCC	RPMI 1640	9.7

EXAMPLE 6. ANTICANCER ACTIVITY *IN VITRO* AGAINST VARIOUS HUMAN

TUMOR CELL LINES FROM THE U.S. NATIONAL CANCER INSTITUTE PANEL

A study measuring the *in vitro* antitumor activity of ECO-04601 was performed by the National Cancer Institute (National Institutes of Health, Bethesda, Maryland, USA) against panel of human cancer cell lines in order to determine the ECO-04601 concentrations needed to obtain a 50% inhibition of cell proliferation (GI_{50}). The operation of this unique screen utilizes 50 different human tumor cell lines, representing leukemia, melanoma and cancers of the lung, colon, brain, ovary, breast, prostate, and kidney.

Culture conditions and plating: The human tumor cell lines of the cancer-screening panel were grown in RPMI 1640 medium containing 5% fetal bovine serum and 2 mM L-glutamine. For a typical screening experiment, cells were inoculated into 96 well microtiter plates in 100 µL at plating densities ranging from 5,000 to 40,000 cells/well depending on the doubling time of individual cell lines (Table 5). After cell inoculation, the microtiter plates were incubated at 37°C, 5% CO_2, 95% air and 100% relative humidity for 24 h prior to addition of experimental drugs. After 24 h, two plates of each cell line were fixed *in situ* with TCA, to represent a measurement of the cell population for each cell line at the time of drug addition (Tz).

Evaluation of inhibitory activity on cell proliferation: ECO-04601 was provided as a lyophilized powder with an estimated purity of 90+%. The compound was stored at -20°C until day of use. ECO-04601 was solubilized in dimethyl sulfoxide at 400-fold the desired final maximum test concentration. At the time of drug addition, an aliquot of frozen concentrate was thawed and diluted to twice the desired final maximum test concentration with complete medium containing 50µg/mL gentamicin. Additional four, 10-fold or ½ log serial dilutions were made to provide a total of five drug concentrations plus control. Aliquots of 100 µl of these different drug dilutions were added to the appropriate microtiter wells already containing 100 µl of medium, resulting in the required final drug concentrations (8.0×10^{-5} M to 8.0×10^{-9} M).

Following drug addition, the plates were incubated for an additional 48 h at 37°C, 5% CO_2, 95% air, and 100% relative humidity. For adherent cells, the assay was terminated by the addition of cold TCA. Cells were fixed *in situ* by the gentle addition of 50 µl of cold 50% (w/v) TCA (final concentration, 10% TCA) and incubated for 60 minutes at 4°C. Supernatants were discarded, and the plates were washed five times with tap water and air-dried. Sulforhodamine B (SRB) solution (100 µl) at 0.4% (w/v) in 1% acetic acid was added to each well, and plates were incubated for 10 minutes at room temperature. After staining, unbound dye was removed by washing five times with 1% acetic acid and the plates were air-dried. Bound stain was subsequently solubilized with 10 mM trizma base, and the absorbance was read on an automated plate reader at a wavelength of 515 nm. For suspension cells, the methodology was the same except that the assay was terminated by fixing settled cells at the bottom of the wells by gently adding 50 µl of 80% TCA (final concentration, 16% TCA).

The growth inhibitory activity of ECO-04601 was measured by NCI utilizing the GI_{50} value, rather than the classical IC_{50} value. The GI_{50} value emphasizes the correction for the cell count at time zero and, using the seven absorbance measurements [time zero, (Tz), control growth, (C), and test growth in the presence of drug at the five concentration levels (Ti)], GI_{50} is calculated as [(Ti-Tz)/(C-Tz)] x 100 = -50, which is the drug concentration resulting in a 50% reduction in the net protein increase (as measured by SRB staining) in control cells during the drug incubation.

Result: ECO-04601 shows a significant antitumor activity against several types of tumor as revealed by the NCI screening. Results of the screen are shown in Table 5, and more detailed results of activity against gliomas are shown in Example 7 (Table 6).

Table 5

Cell Line Name	Type		Inoculation	GI_{50} ($x10^{-6}M$)

		Origin	Density (number of cells/well)	
CCRF-CEM	Leukemia	Human	40,000	1.08
K-562	Leukemia	Human	5,000	1.43
RPMI-8226	Leukemia	Human	20,000	3.15
A549/ATCC	Non-Small Cell Lung	Human	7,500	9.10
EKVX	Non-Small Cell Lung	Human	20,000	0.23
HOP-62	Non-Small Cell Lung	Human	10,000	8.29
NCI-H226	Non-Small Cell Lung	Human	20,000	2.00
NCI-H23	Non-Small Cell Lung	Human	20,000	2.02
NCI-H460	Non-Small Cell Lung	Human	7,500	13.60
NCI-H522	Non-Small Cell Lung	Human	20,000	3.44
COLO 205	Colon	Human	15,000	12.70
HCT-116	Colon	Human	5,000	2.92
HCT-15	Colon	Human	10,000	9.73
HT29	Colon	Human	5,000	20.70
SW-620	Colon	Human	10,000	2.72
SF-268	CNS	Human	15,000	4.94
SF-295	CNS	Human	10,000	12.70
SF-539	CNS	Human	15,000	0.0075
SNB-19	CNS	Human	15,000	2.90
SNB-75	CNS	Human	20,000	7.71
U251	CNS	Human	7,500	2.19
LOX IMVI	Melanoma	Human	7,500	4.53
M14	Melanoma	Human	15,000	4.57
SK-MEL-2	Melanoma	Human	20,000	25.0
SK-MEL-28	Melanoma	Human	10,000	11.6
SK-MEL-5	Melanoma	Human	10,000	7.80
UACC-257	Melanoma	Human	20,000	2.31
UACC-62	Melanoma	Human	10,000	1.55
IGR-OV1	Ovarian	Human	10,000	3.11
OVCAR-3	Ovarian	Human	10,000	13.50
OVCAR-4	Ovarian	Human	15,000	9.67
OVCAR-5	Ovarian	Human	20,000	2.81
OVCAR-8	Ovarian	Human	10,000	2.65
SK-OV-3	Ovarian	Human	20,000	4.00
786-0	Renal	Human	10,000	6.99
A498	Renal	Human	25,000	22.30
ACHN	Renal	Human	10,000	3.10

CAKI-1	Renal	Human	10,000	15.20
RXF 393	Renal	Human	15,000	7.71
SN12C	Renal	Human	15,000	3.85
UO-31	Renal	Human	15,000	19.70
DU-145	Prostate	Human	10,000	3.56
MCF7	Breast	Human	10,000	10.10
NCI/ADR-RES	Breast	Human	15,000	18.30
MDA-MB-231/ATCC	Breast	Human	20,000	2.72
HS 578T	Breast	Human	20,000	2.76
MDA-MB-435	Breast	Human	15,000	15.30
BT-549	Breast	Human	20,000	0.11
T-47D	Breast	Human	20,000	0.77

The results indicate that ECO-04601 was effective against most of the human tumor cell lines that have been assayed in the NCI screening panel suggesting a broad anticancer activity against several types of human cancer.

EXAMPLE 7: IN VITRO ANTIPROLIFERATIVE STUDY AGAINST A PANEL OF GLIOMA CELL LINES

The anticancer activity of ECO-04601 was evaluated using a panel of glioma cancer cell lines shown in Table 6, and the 50% inhibition of cell proliferation (IC_{50}) was determined.

Culture conditions: The cell lines listed in Table 6 were shown to be free of mycoplasma infection and were maintained on DMEM medium supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin-streptomycin, under 5% CO_2 at 37°C. Cells were passaged once a week. Prior to use the cells were detached from the culture flask by treating with trypsin for five to ten minutes. The cells were counted with a Neubauer glass slide and viability assessed by 0.25% trypan blue exclusion. Only flasks with >95% cell viability, were used in the study.

Cell lines amplification and plating: Cells, 5×10^3 cells per well in 100 µL drug-free medium supplemented with 10% serum, were plated in 96-well flat bottom microtiter plates and incubated at 37°C for 48 hrs before treatment.

Evaluation of inhibitory activity on cell proliferation: Cells (in triplicate wells) were incubated 96 hrs with medium containing different concentrations of ECO-04601, starting at 5.0 µg/ml (10 µM). The compound was used in a solution of 1% DMSO in D-MEM or RPMI media (or other equivalent media). The concentrations of ECO-04601 were as follows: 10 µM (5.0 µg/ml), 1 µM (0.50 µg/ml), 0.5 µM (0.25 µg/ml), 0.1 µM (0.050 µg/ml), 0.5 µM (0.025 µg/ml), 0.01 µM (0.0050 µg/ml), 0.001 µM (0.00050 µg/ml). Negative controls were cells treated with vehicle alone (1% DMSO in culture medium). Positive controls were cells treated with 4 to 6 increasing concentrations of cisplatin (CDDP) (data not shown). The optical density was measured before incubation (time 0) and following 96 hrs of incubation with test compound in order to measure the growth rate of each cell line.

At the end of the cell treatment, cell culture media was replaced with 150 µl of fresh medium containing 10 mM of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, pH 7.4. Then 50 µl of 2.5 mg/ml of 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide in PBS pH 7.4, were added to each well and the culture plates incubated for 4 hrs at 37°C. The resulting supernatant was removed and formazan crystals were dissolved with 200 µl of DMSO followed by 25 µl of glycine buffer (0.1 M glycine plus 0.1 M NaCl, pH 10.5). The optical density was read in each well using a single wavelength spectrophotometer plate reader at 570 nm. Results were expressed as the concentration of drug, which inhibits 50% of the cell growth (IC_{50}). Each of the cell lines was tested in at least 3 independent experiments.

Results shown in Table 6 confirmed the activity of ECO-04601 against different brain cancer cell lines including gliosarcoma, which is the most malignant form of type IV glioblastoma multiform. Gliosarcomas are a mixture of glial and endothelial cells and are resistant to any chemotherapy.

Table 6

Cell lines	Type	Origin	Source	IC_{50} ($\times 10^{-6}$ M)
9L	Gliosarcoma	Rat	ATCC	6.82 ± 2.90
GHD	Astrocytoma	Human	ATCC	6.29 ± 2.98
U 373	Astrocytoma	Human	ATCC	3.83 ± 1.37
GL26	Glioblastoma	Human	ATCC	8.93 ± 1.10
C6	Glioblastoma	Rat	ATCC	4.28 ± 2.82
DN	Oligodendroglioma	Human	ATCC	3.26 ± 0.93
GHA	Oligodendroglioma	Human	ATCC	1.78 ± 0.84

EXAMPLE 8: EFFECT ON THE ENZYMATIC ACTIVITY OF HUMAN LIPOXYGENASE (5-LO)

5-Lipoxygenase catalyzes the oxidative metabolism of arachidonic acid to 5-hydroxyeicosatetraenoic acid (5-HETE), the initial reaction leading to formation of leukotrienes. Eicosanoids derived from arachidonic acid by the action of lipoxygenases or cycloxygenases have been found to be involved in acute and chronic inflammatory diseases (i.e. asthma, multiple sclerosis, rheumatoid arthritis, ischemia, edema) as well in neurodegeneration (Alzheimer disease), aging and various steps of carcinogenesis, including tumor promotion, progression and metastasis.

The aim of this study was to determine whether ECO-04601, is able to block the formation of leukotrienes by inhibiting the enzymatic activity of human 5-LO. Methods employed are based on Carter et al (1991) J. Pharmacol. Exp. Ther. 256(3):929-937, and Safayhi (2000), Planta Medica 66:110-113 which are incorporated herein in their

entirety by reference.

Experimental Design: Human peripheral blood mononuclear cells (PMNs) were isolated through a Ficoll-Paque density gradient. PMNs were stimulated by addition A23187 (30 µM final concentration). Stimulated PMNs were adjusted to a density of 5 $x10^6$ cells/mL in HBBS medium and incubated with the vehicle control (DMSO), ECO-04601 (at final concentrations of 0.1, 0.5, 1, 2.5, 5 and 10 µM) and NDGA as positive control (at final concentrations of 3, 1, 0.3, 0.1 and 0.03 µM) for 15 minutes at 37°C. Following incubation, samples were neutralized with NaOH and centrifuged. Leukotriene B4 content was measured in the supernatant using an Enzyme Immunosorbant Assay (EIA) assay.

Results: Results shown in *Figure 8* demonstrated that ECO-04601 inhibited the activity of human 5-LO with an apparent IC_{50} = 0.93 µM (versus 0.1 µM for the positive control NDGA) and therefore displays anti-inflammatory properties.

EXAMPLE 9: *IN VIVO* EFFICACY IN A GLIOMA MODEL

The aim of this study was to test whether ECO-04601 administered by i.p. route prevents or delays tumor growth in C6 glioblastoma cell-bearing mice, and to determine an effective dosage regimen.

Animals: A total of 60 six-week-old female mice (*Mus musculus* nude mice), ranging between 18 to 25 g in weight, were observed for 7 days before treatment. Animal experiments were performed according to ethical guidelines of animal experimentation (*Charte du comité d'éthique du CNRS, juillet 2003*) and the English guidelines for the welfare of animals in experimental neoplasia (*WORKMAN, P., TWENTYMAN, P., BALKWILL, F., et al. (1998). United Kingdom Coordinating Committee on Cancer Research (UKCCCR) Guidelines for the welfare of animals in experimental neoplasia*

(Second Edition, July 1997; British Journal of Cancer ***77****:1-10*). Any dead or apparently sick mice were promptly removed and replaced with healthy mice. Sick mice were euthanized upon removal from the cage. Animals were maintained in rooms under controlled conditions of temperature (23±2°C), humidity (45±5%), photoperiodicity (12 hrs light / 12 hrs dark) and air exchange. Animals were housed in polycarbonate cages (5/single cage) that were equipped to provide food and water. Animal bedding consisted of sterile wood shavings that were replaced every other day. Food was provided *ad libitum,* being placed in the metal lid on the top of the cage. Autoclaved tap water was provided *ad libitum.* Water bottles were equipped with rubber stoppers and sipper tubes. Water bottles were cleaned, sterilized and replaced once a week. Two different numbers engraved on two earrings identified the animals. Each cage was labelled with a specific code.

Tumor Cell Line: The C6 cell line was cloned from a rat glial tumor induced by N-nitrosomethyurea (NMU) by Premont et al. (*Premont J, Benda P, Jard S., [3H] norepinephrine binding by rat glial cells in culture. Lack of correlation between binding and adenylate cyclase activation. Biochim Biophys Acta. 1975 Feb 13;381(2):368-76.*) after series of alternate culture and animal passages.

Cells were grown as adherent monolayers at 37°C in a humidified atmosphere (5% CO_2, 95% air). The culture medium was DMEM supplemented with 2 mM L-glutamine and 10% fetal bovine serum. For experimental use, tumor cells were detached from the culture flask by a 10 min treatment with trypsin-versen. The cells were counted in a hemocytometer and their viability assessed by 0.25% trypan blue exclusion.

Preparation of the Test Article: For the test article, the following procedure was followed for reconstitution (performed immediately preceding injection). The vehicle consisted of a mixture of benzyl alcohol (1.5%), ethanol (8.5%), propylene glycol (27%), PEG 400 (27%), dimethylacetamide (6%) and water (30%). The vehicle solution was first vortexed in order to obtain a homogeneous liquid. 0.6 mL of the vortexed vehicle

solution was added to each vial containing the test article (ECO-04601). Vials were mixed thoroughly by vortexing for 1 minute and inverted and shaken vigorously. Vials were mixed again prior to injection into each animal.

Animal Inoculation with tumor cells: Experiment started at day 0 (D_0). On D_0, mice received a superficial intramuscular injection of C6 tumor cells (5×10^5 cells) in 0.1 mL of DMEM complete medium into the upper right posterior leg.

Treatment regimen and Results

In a first series of experiments, treatment started 24 hrs following inoculation of C6 cells. On the day of the treatment, each mouse was slowly injected with 100 µL of test or control articles by i.p. route. For all groups, treatment was performed until the tumor volume of the saline-treated mice (group 1) reached approximately 3 cm^3 (around day 16). Mice of group 1 were treated daily with a saline isosmotic solution for 16 days. Mice of group 2 were treated daily with the vehicle solution for 16 days. Mice of group 3 were treated daily with 10 mg/kg of ECO-04601 for 16 days. Mice of group 3 were treated every two days with 30 mg/kg of ECO-04601 and received 8 treatments. Mice of group 5 were treated every three days with 30 mg/kg of ECO-04601 and received 6 treatments. Measurement of tumor volume started as soon as tumors became palpable (>100 mm^3; around day 11 post-inoculation) and was evaluated every second day until the end of the treatment using callipers. As shown in Table 7 and Figure 9, the mean value of the tumor volume of all ECO-04601 treated groups (6 mice/group) was significantly reduced as demonstrated by the one-way analysis of variance (Anova) test followed by the non-parametric Dunnett's multiple comparison test comparing treated groups to the saline group. An asterisk in the P value column of Table 7 indicates a statistically significant value, while "ns" signifies not significant.

Table 7

Treatment	Treatment	Tumor volume	%	P

	regimen	(mm^3) (mean ± SEM)	Inhibition	value
Saline	Q1 x 16	3,004.1 ± 249.64	-	-
Vehicle solution	Q1 x 16	2,162.0 ± 350.0	28.0%	>0.05 ns
ECO-04601 (10 mg/kg)	Q1 x 16	1,220.4 ± 283.46	59.4%	<0.01 *
ECO-04601 (30 mg/kg)	Q2 x 8	1,236.9 ± 233.99	58.8%	<0.01 *
ECO-04601 (30 mg/kg)	Q3 x 6	1,184.1 ± 221.45	60.6%	<0.01 *

In a second series of experiments, treatment started at day 10 following inoculation of C6 cells when tumors became palpable (around 100 to 200 mm^3). Treatment was repeated daily for 5 consecutive days. On the day of the treatment, each mouse was slowly injected with 100 µL of ECO-04601 by i.p. route. Mice of group 1 were treated daily with saline isosmotic solution. Mice of group 2 were treated daily with the vehicle solution. Mice of group 3 were treated daily with 20 mg/kg of ECO-04601. Mice of group 4 were treated daily with 30 mg/kg of ECO-04601. Mice were treated until the tumor volume of the saline-treated control mice (group 1) reached around 4 cm^3. Tumor volume was measured every second day until the end of the treatment using callipers. As shown in Table 8 and *Figure 10*, the mean value of the tumor volume of all ECO-04601 treated groups (6 mice/group) was significantly reduced as demonstrated by the one-way analysis of variance (Anova) test followed by the non-parametric Dunnett's multiple comparison test comparing treated groups to the saline group. An asterisk in the P value column of Table 8 indicates a statistically significant value, while "ns" signifies not statistically significant.

Histological analysis of tumor sections showed pronounced morphological changes between ECO-04601-treated tumors and control groups. In tumors treated with ECO-04601 (20 – 30 mg/kg), cell density was decreased and the nuclei of remaining tumor cells appeared larger and pycnotic while no such changes were observed for vehicle-treated mice (*Figure 11*).

Table 8

Treatment	Treatment regimen	Tumor volume (mm^3) (mean ± SEM)	% Inhibition	P value
Saline	Q1 x 5	4,363.1 ± 614.31	-	-
Vehicle solution	Q1 x 5	3,205.0 ± 632.37	26.5%	>0.05 ns
ECO-04601 (20 mg/kg)	Q1 x 5	1,721.5 ± 374.79	60.5%	<0.01 *
ECO-04601 (30 mg/kg)	Q1 x 5	1,131.6 ± 525.21	74.1%	<0.01 *

EXAMPLE 10: GENERATION OF VARIANTS OF ECO-04601 ACCORDING TO THE INVENTION

Variants of the ECO-04601 molecule, for example those identified herein as Formulae III-LIX, can be generated by standard organic chemistry approaches. General principles of organic chemistry required for making and manipulating the compounds described herein, including functional moieties, reactivity and common protocols are described, for example, in "Advanced Organic Chemistry," 3rd Edition by Jerry March (1985) which is incorporated herein by reference in its entirety. In addition,

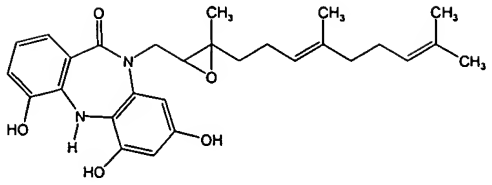
it will be appreciated by one of ordinary skill in the art that the synthetic methods described herein may use a variety of protecting groups, whether or not they are explicitly described. A "protecting group" as used herein means a moiety used to block one or more functional moieties such as reactive groups including oxygen, sulfur or nitrogen, so that a reaction can be carried out selectively at another reactive site in a polyfunctional compound. General principles for the use of protective groups, their applicability to specific functional groups and their uses are described for example in T. H. Greene and P. G. M. Wuts, Protective Groups in Organic Synthesis, 3rd Edition, John Wiley & Sons, New York (1999).

Scheme 1: Epoxide variants

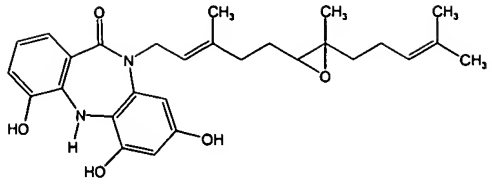
The epoxide compounds of the present invention (e.g., compounds according to exemplary Formulae VII-XIV) are made from the compound of Formula II (ECO-04601) by treatment with any of a number of epoxidizing reagents such as perbenzoic acid, monoperphthalic acid or more preferably by m-chloroperbenzoic acid in an inert solvent such as tetrahydrofuran (THF) dichloromethane or 1,2-dichloroethane. It will be appreciated by one of ordinary skill in the art that slightly greater than one molecule equivalent of epoxidizing agent will result in the maximal yield of mono-epoxides, and that the reagent, solvent, concentration and temperature of the reaction will dictate the ratio of specific mono-epoxides formed. It will also be appreciated that the mono-epoxides will be enantiomeric mixtures, and that the di-epoxides and the tri-epoxide can be prepared as diastereomers and that the conditions of the reaction will determine the ratios of the products. One skilled in the art will appreciate that under most conditions of reactions the product will be a mixture of all possible epoxides and that these may be separated by standard methods of chromatography. Exemplary approaches to the generation of mono-, di-, and tri-epoxides are provided below.

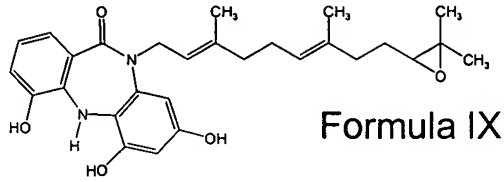
A) Mono-epoxides of the Formulae VII, VIII, and IX by epoxidation of the compound of Formula II:

Formula VII



Formula VIII



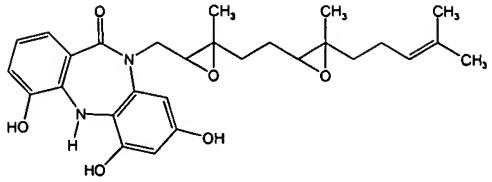


Formula IX

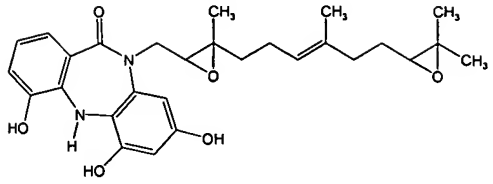
To a solution of the compound of Formula II dissolved in tetrahydrofuran (THF) is added 1.1 equivalents of *meta*-chloroperbenzoic acid. The reaction is cooled in an ice bath and stirred at 0 °C for 1-2 hours. The reaction mixture is then evaporated to dryness, re-dissolved in methanol and subjected to liquid chromatography on a column of Sephadex LH-20 to isolate a mixture of predominantly the compounds of Formulae VII, VIII and IX, contaminated with some unchanged starting material and some di- and tri- epoxides. The compounds of Formulae VII, VIII and XIX are separated and purified by HPLC using the system described in Example 2 for the purification of the compound of Formulae II. In a typical experiment yields of 15% to 25% are obtained for each of the compounds of Formulae VII, VIII and IX.

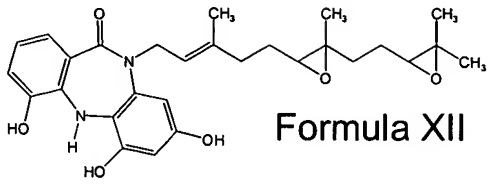
B) Synthesis of Compounds of Formulae X, XI, and XII by di-epoxidation of Compound of Formula II:

Formula X



Formula XI

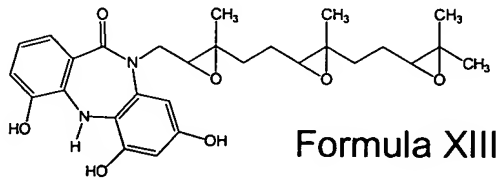




Formula XII

To a solution of the compound of Formula II dissolved in tetrahydrofuran (THF) is added 2.3 equivalents of *meta*-chloroperbenzoic acid. The reaction is cooled in an ice bath and stirred at 0 °C for 1-2 hours. The reaction mixture is then evaporated to dryness, re-dissolved in methanol and subjected to liquid chromatography on a column of Sephadex LH-20 to isolate a mixture of predominantly the compounds of Formulae X, XI and XII, contaminated with traces of unchanged starting material and some mono- and tri- epoxides. The Compounds of Formulae X, XI and XII are separated and purified by HPLC using the system described in Example 2 for the purification of the compound of Formulae II. In a typical experiment, yields of 15% to 20% are obtained for each of the compounds of Formulae X, XI and XII.

C) Synthesis of Compound of Formula XIII by tri-epoxidation of Compound of Formula II:

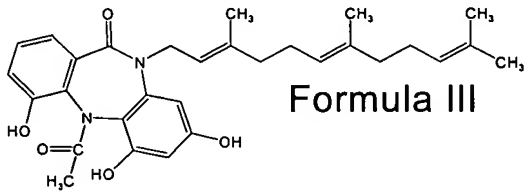


Formula XIII

To a solution of the compound of Formula II, dissolved in tetrahydrofuran (THF), is added 3.5 equivalents of *meta*-chloroperbenzoic acid. The reaction is cooled in an

ice bath and stirred at 0 °C for 1-2 hours. The reaction mixture is then evaporated to dryness, re-dissolved in methanol and subjected to liquid chromatography on a column of Sephadex LH-20 to isolate the compound of Formula XIII as a mixture of diasteriomers in a yield of 80+%.

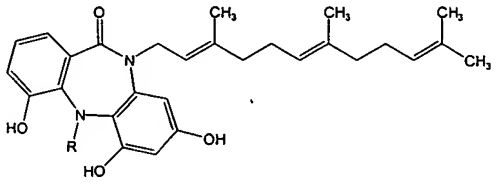
Scheme 2: Synthesis of Compound of Formula III by N-acetylation of Compound of Formula II.



Formula III

To a solution of Compound of Formula II dissolved in tetrahydrofuran (THF) is added 1.2 equivalents of acetic anhydride and a few drops of triethylamine. The reaction mixture allowed to stand at room temperature for 1-2 hours and then evaporated to dryness under reduced pressure to obtain the Compound of Formula III in an essentially pure form in an almost quantitative yield

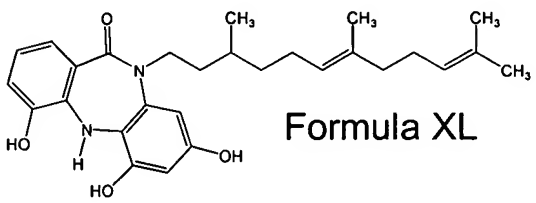
Scheme 3: Syntheses of Compounds of Formulae IV and V by N-alkylation of Compound of
Formula II.



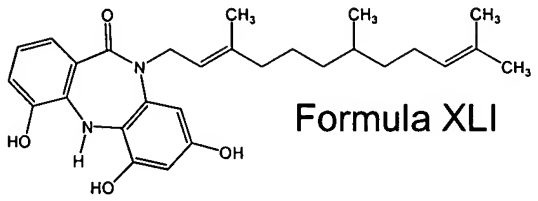
Formula IV R = benzyl
Formula V R = ethyl

To a solution of Compound of Formula II dissolved in terachloroethylene is added 1.2 equivalents of the appropriate alkyl bromide (benzyl bromide for the compound of formula IV or ethyl bromide for the Compound of Formula V). The reaction mixture the reaction mixture is heated under reflux for 1-2 hours and then evaporated to dryness under reduced pressure to obtain the Compound of Formula IV or the Compound of Formula V respectively, in an essentially pure form in an almost quantitative yield.

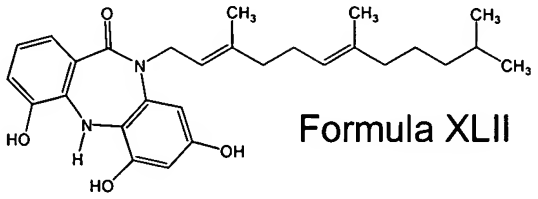
Scheme 4: Syntheses of Compounds of Formulae XL, XLI and XLII by catalytic reduction of Compound of Formula II.



Formula XL



Formula XLI

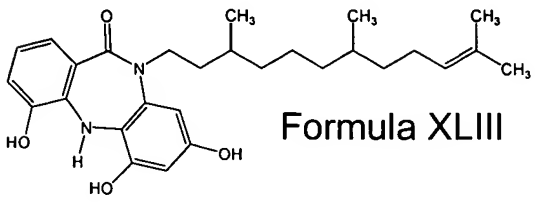


Formula XLII

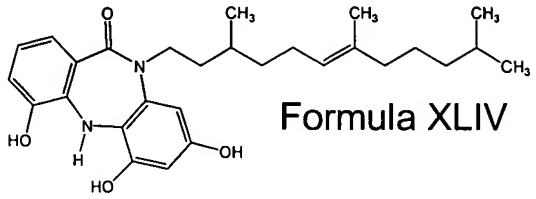
A solution of the Compound of Formula II (462 mg) in ethanol (200 ml) with palladium on charcoal (25 mg of 5%) is shaken in an hydrogenation apparatus in an atmosphere of hydrogen. The uptake of hydrogen by the reaction is measured carefully and at the point where one millimole of hydrogen has been consumed, shaking is stopped, the vessel is rapidly evacuated and the atmosphere is replaced with nitrogen. The catalyst is removed by filtration and the filtrate is concentrated to obtain a crude mixture of the Compounds of Formulae XL, XLI and XLII contaminated by unreacted starting material and minor amounts of over reduced products. The desired products may be separated and purified by HPLC or HSCC chromatography using the systems as described in Example 2 above, to obtain approximately 100 mg of each of the Compounds of Formulae XL, XLI and XLII.

Scheme 5: Syntheses of Compounds of Formulae XLIII, XLIV and XLV by catalytic reduction of

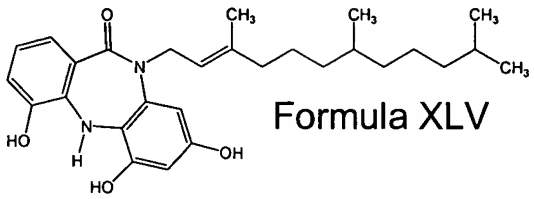
Compound of Formula II.



Formula XLIII



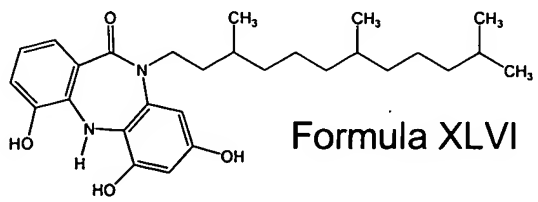
Formula XLIV



Formula XLV

A solution of the Compound of Formula II (462 mg) in ethanol (200 ml) with palladium on charcoal (25 mg of 5%) is shaken in an hydrogenation apparatus in an atmosphere of hydrogen. The uptake of hydrogen by the reaction is measured carefully and at the point where two millimoles of hydrogen has been consumed, shaking is stopped, the vessel is rapidly evacuated and the atmosphere is replaced with nitrogen. The catalyst is removed by filtration and the filtrate is concentrated to obtain a crude mixture of the Compounds of Formulae XLIII, XLIV and XLV contaminated by trace amounts unreacted starting material and minor amounts of under and over reduced products. The desired products may be separated and purified by HPLC or HSCC chromatography using the systems as described in Example 2 above, to obtain approximately 100 mg of each of the Compounds of Formulae XLIII, XLIV and XLV.

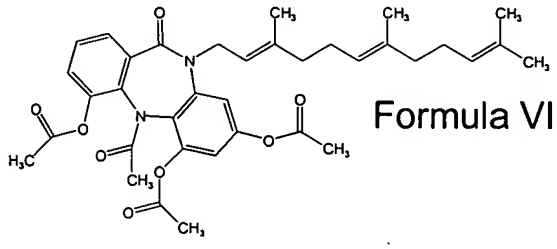
Scheme 6: Syntheses of Compound of Formula XLVI by catalytic reduction of Compound of Formula II.



Formula XLVI

A solution of the Compound of Formula II (462 mg) in ethanol (200 ml) with palladium on charcoal (25 mg of 5%) is shaken in an hydrogenation apparatus in an atmosphere of hydrogen. The uptake of hydrogen by the reaction is measured carefully and at the point where three millimoles of hydrogen has been consumed, shaking is stopped, the vessel is rapidly evacuated and the atmosphere is replaced with nitrogen. The catalyst is removed by filtration and the filtrate is concentrated to obtain an essentially pure sample of the Compound of Formula XLVI

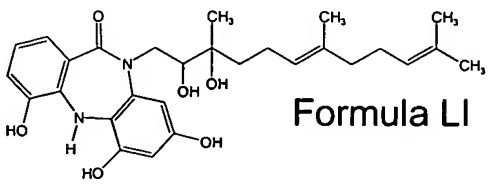
Scheme 7: Syntheses of Compound of Formula VI by peracetylation of Compound of Formula II.



Formula VI

A solution of the Compound of Formula II (100 mg) in acetic anhydride (5 ml) is treated with pyridine (250 ul). The reaction mixture is allowed to stand overnight at room temperature and is then diluted with toluene (100 ml). The toluene solution is washed well with aqueous 5% sodium bicarbonate solutions, then with water and is finally concentrated under reduced pressure to give an essentially pure sample of the Compound of Formula VI in almost quantitative yield.

Scheme 8: Syntheses of Compound of Formula LI by opening the epoxide of Compound of Formula VII.

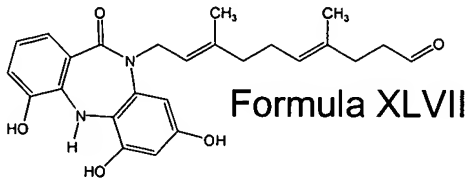


Formula LI

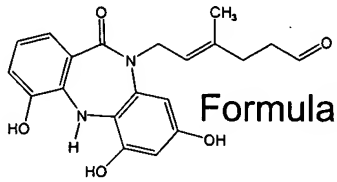
A solution of the Compound of Formula VII (100 mg) in tetrahydrofuran (50 ml) is treated with 1N aqueous hydrochloric acid (5 ml). The reaction mixture is stirred

overnight at room temperature and is then diluted with toluene (100 ml) and water (200 ml). The toluene layer is separated and the aqueous layer is extracted with a further 100 ml of toluene. The combined toluene layers are washed once more with water (50 ml) and the separated and dried under vacuum to give the vicinal glycol Compound of Formula LI.

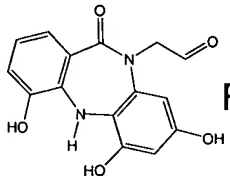
Scheme 9: Syntheses of Compounds of Formulae XLVII, XLIX and LI by ozonolysis of Compound of Formula II.



Formula XLVII



Formula XLIX



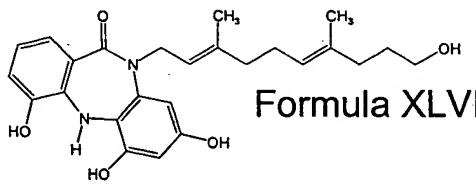
Formula LI

A solution of the Compound of Formula II (462 mg) in dry ethyl acetate (200 ml) in an ozonolysis apparatus is cooled to below –20°C. A stream of ozone-containing oxygen is passed into the solution from an ozone generator, which has been precalibrated such that the rate of ozone generation is known. To obtain predominantly the compound of Formula XLVII the passage of ozone is halted after 0.9 millimole have been generated. To obtain predominantly the compound of Formula XLIX the ozone passage is halted after 2 millimoles have been generated and to obtain the compound of Formula LI as the predominant product 3.3 millimoles of ozone are generated.

At the completion of the ozonolysis, the reaction mixture is transferred to an hydrogenation apparatus, 5% palladium on calcium carbonate catalyst (0.2 g) is added to the reaction mixture which is maintained at less than –20°C and is hydrogenated. When hydrogen uptake is complete the hydrogen atmosphere is replaced with nitrogen and the reaction mixture is allowed to come to room temperature, filtered to remove

catalyst and the filtrate is concentrated. The crude product may be purified by chromatography using either HPLC or HSCC with the systems as described in Example 2 to give, dependent on the amount of ozone used, Compounds of Formulae XLVII, XLIX and LI.

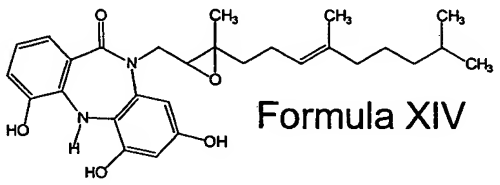
Scheme 10: Synthesis of Compound of Formulae XLVIII by reduction of the aldehyde of Compound of Formula XLVII.



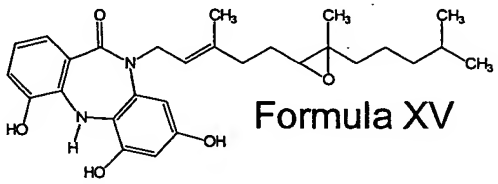
Formula XLVIII

A solution of the Compound of Formula XLVIII (50 mg) in isopropanol (5 ml) is cooled in an ice-salt bath and sodium borohydride (10 mg) is added and the mixture is stirred for 20 minutes. It is then diluted with water (20 ml) and extracted twice with toluene (10 ml portions) at ambient temperature. The combined toluene extracts are filtered and the filtrate is concentrated to give the Compound of Formula XLVII.

Scheme 11: Syntheses of Compounds of Formulae XIV and XV by epoxidation of the Compound of Formula XLII.



Formula XIV

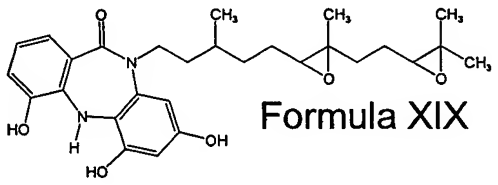


Formula XV

To a solution of Compound of Formula XLII dissolved in tetrahydrofuran (THF) is added 1.1 equivalents of *meta*-chloroperbenzoic acid. The reaction is cooled in an ice bath and stirred at 0 °C for 1-2 hours. The reaction mixture is then evaporated to dryness, re-dissolved in methanol and subjected to liquid chromatography on a column of Sephadex LH-20 to isolate a mixture of predominantly the Compounds of Formulae XIV, and XV, contaminated with some unchanged starting material and some

diepoxide. The Compounds of Formulae XIV and XV are separated and purified by HPLC or HSCC using one of the systems described in Example 2 for the purification of the Compound of Formulae II. In a typical experiment yields of 35% to 40% are obtained for each of the Compounds of Formulae XIV and XV.

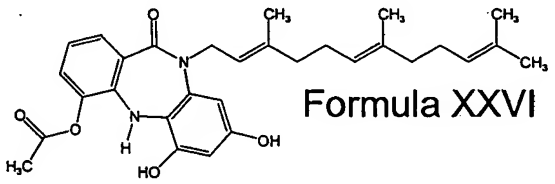
Scheme 12: Synthesis of Compound of Formulae XIX by epoxidation of the Compound of Formula XL.



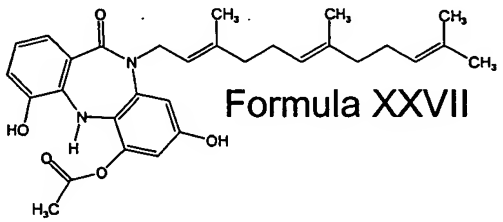
Formula XIX

To a solution of Compound of Formula XL dissolved in tetrahydrofuran (THF) is added 2.2 equivalents of *meta*-chloroperbenzoic acid. The reaction is cooled in an ice bath and stirred at 0 °C for 1-2 hours. The reaction mixture is then evaporated to dryness, re-dissolved in methanol and subjected to liquid chromatography on a column of Sephadex LH-20 to isolate essentially pure Compound of Formulae XIX in good yield.

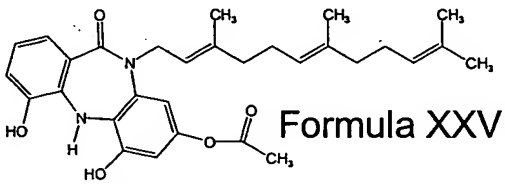
Scheme 13: Syntheses of Compounds of Formulae XXVI, XXVII and XXVIII by esterification of the Compound of Formula II.



Formula XXVI



Formula XXVII

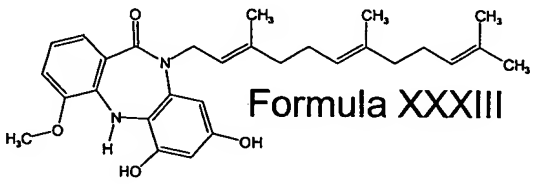


Formula XXVIII

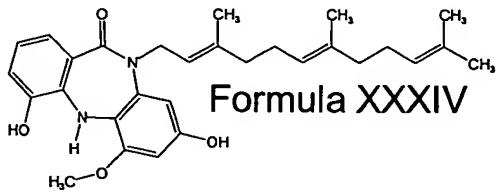
To a solution of Compound of Formula II dissolved in toluene (9 parts) tetrahydrofuran (1 part), cooled in an ice-bath is added 1.1 equivalents of acetic anhydride and two drops of boron trifluoride etherate. The reaction is maintained cool in an ice bath and stirred at 0 °C for 1-2 hours. The reaction mixture is then poured into

aqueous 5% sodium bicarbonate solution shaken and the toluene layer is removed. The aqueous layer is re-extracted with toluene and the combined toluene layers are concentrated to a mixture of predominantly the Compounds of Formulae XXVI, XXVII and XXVIII, contaminated with some unchanged starting material and some diacetates. The Compounds of Formulae XXVI, XXVII and XXVIII are separated and purified by HPLC or HSCC using one of the systems described in Example 2 for the purification of the Compound of Formulae II. In a typical experiment yields of 25% to 30% are obtained for each of the Compounds of Formulae XXVI, XXVII and XXVIII.

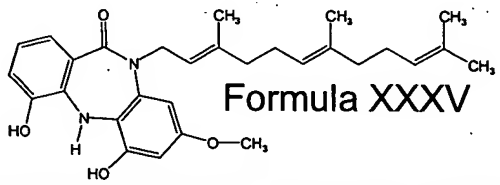
Scheme 14: Syntheses of Compounds of Formulae XXXIII, XXXIV and XXXV by methylation of the Compound of Formula II.



Formula XXXIII



Formula XXXIV



Formula XXXV

A solution of the Compound of Formula II (1 g) in tetrahydrofuran 50 (ml) is titrated with exactly one equivalent of sodium methoxide, allowed to stand for 30 minutes at room temperature and then treated with 1.2 equivalents of dimethylsulphate. Heat the mixture under reflux for one hour, cool to room temperature and pour into a mixture of toluene (200 ml) and water (200 ml). The layers are separated and the aqueous layer is extracted once more with an equal portion of toluene. The combined toluene layers are washed once with 1N aqueous acetic acid and then concentrated to s crude product, which is predominantly a mixture of the Compounds of Formulae XXXIII, XXXIV and XXXV with some unchanged starting material and traces of over-methylated derivatives. The desired products may be separated and purified by HPLC or HSCC chromatography using the systems as described in Example 2 above, to obtain approximately 200 mg of each of the Compounds of Formulae XXXIII, XXXIV

or HSCC chromatography using the systems as described in Example 2 above, to obtain approximately 200 mg of each of the Compounds of Formulae XXXIII, XXXIV and XXXV.

EXAMPLE 11: GENES AND PROTEINS FOR THE PRODUCTION OF COMPOUNDS OF FORMULA

Micromonospora sp. strain 046-ECO11 is a representative microorganism useful in the production of the compound of the invention. Strain 046-ECO11 has been deposited with the International Depositary Authority of Canada (IDAC), Bureau of Microbiology, Health Canada, 1015 Arlington Street, Winnipeg, Manitoba, Canada R3E 3R2 on March 7, 2003 and was assigned IDAC accession no. 070303-01. The biosynthetic locus for the production of the compound of Formula II was identified in the genome of *Micromonospora sp.* strain 046-ECO11 using the genome scanning method described in USSN 10/232,370, CA 2,352,451 and Zazopoulos *et. al.*, *Nature Biotechnol.*, 21, 187-190 (2003).

The biosynthetic locus spans approximately 52,400 base pairs of DNA and encodes 43 proteins. More than 10 kilobases of DNA sequence were analyzed on each side of the locus and these regions were deemed to contain primary genes or genes unrelated to the synthesis of the compound of Formula II. As illustrated in FIGURE 12, the locus is contained within three sequences of contiguous base pairs, namely Contig 1 having the 36,602 contiguous base pairs of SEQ ID NO: 1 and comprising ORFs 1 to 31 (SEQ ID NOS: 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61 and 63), Contig 2 having the 5,960 contiguous base pairs of SEQ ID NO: 64 and comprising ORFs 32 to 35 (SEQ ID NOS: 66, 68, 70 and 72), and Contig 3 having the 9,762 base pairs of SEQ ID NO: 73 and comprising ORFs 36 to 43 (SEQ ID NOS: 75, 77, 79, 81, 83, 85, 87 and 89). The order, relative position and orientation of the 43 open reading frames representing the proteins of the biosynthetic locus are illustrated schematically in FIGURE 12. The top line in FIGURE 12 provides a scale in base pairs. The gray bars depict the three DNA

contigs (SEQ ID NOS: 1, 64 and 73) that cover the locus. The empty arrows represent the 43 open reading frames of this biosynthetic locus. The black arrows represent the two deposited cosmid clones covering the locus.

The biosynthetic locus will be further understood with reference to the sequence listing which provides contiguous nucleotide sequences and deduced amino acid sequences of the locus from *Micromonospora sp.* strain 046-ECO11. The contiguous nucleotide sequences are arranged such that, as found within the biosynthetic locus, Contig 1 (SEQ ID NO: 1) is adjacent to the 5' end of Contig 2 (SEQ ID NO: 64), which in turn is adjacent to Contig 3 (SEQ ID NO: 73). The ORFs illustrated in FIGURE 12 and provided in the sequence listing represent open reading frames deduced from the nucleotide sequences of Contigs 1, 2 and 3 (SEQ ID NOS: 1, 64 and 73). Referring to the Sequence Listing, ORF 1 (SEQ ID NO: 3) is the polynucleotide drawn from residues 2139 to 424 of SEQ ID NO: 1, and SEQ ID NO: 2 represents that polypeptide deduced from SEQ ID NO: 3. ORF 2 (SEQ ID NO: 5) is the polynucleotide drawn from residues 2890 to 4959 of SEQ ID NO: 1, and SEQ ID NO: 4 represents the polypeptide deduced from SEQ ID NO: 5. ORF 3 (SEQ ID NO: 7) is the polynucleotide drawn from residues 7701 to 5014 of SEQ ID NO: 1, and SEQ ID NO: 6 represents the polypeptide deduced from SEQ ID NO: 7. ORF 4 (SEQ ID NO: 9) is the polynucleotide drawn from residues 8104 to 9192 of SEQ ID NO: 1, and SEQ ID NO: 8 represents the polypeptide deduced from SEQ ID NO: 9. ORF 5 (SEQ ID NO: 11) is the polynucleotide drawn from residues 9192 to 10256 of SEQ ID NO: 1, and SEQ ID NO: 10 represents the polypeptide deduced from SEQ ID NO: 11. ORF 6 (SEQ ID NO: 13) is the polynucleotide drawn from residues 10246 to 11286 of SEQ ID NO: 1, and SEQ ID NO: 12 represents the polypeptide deduced from SEQ ID NO: 13. ORF 7 (SEQ ID NO: 15) is the polynucleotide drawn from residues 11283 to 12392 of SEQ ID NO: 1, and SEQ ID NO: 14 represents the polypeptide deduced from SEQ ID NO: 15. ORF 8 (SEQ ID NO: 17) is the polynucleotide drawn from residues 12389 to 13471 of SEQ ID NO: 1, and SEQ ID NO: 16 represents the polypeptide deduced from SEQ ID NO: 17. ORF 9 (SEQ ID NO: 19) is the polynucleotide drawn from residues 13468 to 14523 of SEQ ID NO: 1,

and SEQ ID NO: 18 represents the polypeptide deduced from SEQ ID NO: 19. ORF 10 (SEQ ID NO: 21) is the polynucleotide drawn from residues 14526 to 15701 of SEQ ID NO: 1, and SEQ ID NO: 20 represents the polypeptide deduced from SEQ ID NO: 21. ORF 11 (SEQ ID NO: 23) is the polynucleotide drawn from residues 15770 to 16642 of SEQ ID NO: 1, and SEQ ID NO: 22 represents the polypeptide deduced from SEQ ID NO: 23. ORF 12 (SEQ ID NO: 25) is the polynucleotide drawn from residues 16756 to 17868 of SEQ ID NO: 1, and SEQ ID NO: 24 represents the polypeptide deduced from SEQ ID NO: 25. ORF 13 (SEQ ID NO: 27) is the polynucleotide drawn from residues 17865 to 18527 of SEQ ID NO: 1, and SEQ ID NO: 26 represents the polypeptide deduced from SEQ ID NO: 27. ORF 14 (SEQ ID NO: 29) is the polynucleotide drawn from residues 18724 to 19119 of SEQ ID NO: 1, and SEQ ID NO: 28 represents the polypeptide deduced from SEQ ID NO: 29. ORF 15 (SEQ ID NO: 31) is the polynucleotide drawn from residues 19175 to 19639 of SEQ ID NO: 1, and SEQ ID NO: 30 represents the polypeptide deduced from SEQ ID NO: 31. ORF 16 (SEQ ID NO: 33) is the polynucleotide drawn from residues 19636 to 21621 of SEQ ID NO: 1, and SEQ ID NO: 32 represents the polypeptide deduced from SEQ ID NO: 33. ORF 17 (SEQ ID NO: 35) is the polynucleotide drawn from residues 21632 to 22021 of SEQ ID NO: 1, and SEQ ID NO: 34 represents the polypeptide deduced from SEQ ID NO: 35. ORF 18 (SEQ ID NO: 37) is the polynucleotide drawn from residues 22658 to 22122 of SEQ ID NO: 1, and SEQ ID NO: 36 represents the polypeptide deduced from SEQ ID NO: 37. ORF 19 (SEQ ID NO: 39) is the polynucleotide drawn from residues 24665 to 22680 of SEQ ID NO: 1, and SEQ ID NO: 38 represents the polypeptide deduced from SEQ ID NO: 39. ORF 20 (SEQ ID NO: 41) is the polynucleotide drawn from residues 24880 to 26163 of SEQ ID NO: 1, and SEQ ID NO: 40 represents the polypeptide deduced from SEQ ID NO: 41. ORF 21 (SEQ ID NO: 43) is the polynucleotide drawn from residues 26179 to 27003 of SEQ ID NO: 1, and SEQ ID NO: 42 represents the polypeptide deduced from SEQ ID NO: 43. ORF 22 (SEQ ID NO: 45) is the polynucleotide drawn from residues 27035 to 28138 of SEQ ID NO: 1, and SEQ ID NO: 44 represents the polypeptide deduced from SEQ ID NO: 45. ORF 23 (SEQ ID NO: 47) is the polynucleotide drawn from residues 28164 to 28925 of SEQ ID NO: 1, and SEQ ID NO:

46 represents the polypeptide deduced from SEQ ID NO: 47. ORF 24 (SEQ ID NO: 49) is the polynucleotide drawn from residues 28922 to 30238 of SEQ ID NO: 1, and SEQ ID NO: 48 represents the polypeptide deduced from SEQ ID NO: 49. ORF 25 (SEQ ID NO: 51) is the polynucleotide drawn from residues 30249 to 31439 of SEQ ID NO: 1, and SEQ ID NO: 50 represents the polypeptide deduced from SEQ ID NO: 51. ORF 26 (SEQ ID NO: 53) is the polynucleotide drawn from residues 31439 to 32224 of SEQ ID NO: 1, and SEQ ID NO: 52 represents the polypeptide deduced from SEQ ID NO: 53. ORF 27 (SEQ ID NO: 55) is the polynucleotide drawn from residues 32257 to 32931 of SEQ ID NO: 1, and SEQ ID NO: 54 represents the polypeptide deduced from SEQ ID NO: 55. ORF 28 (SEQ ID NO: 57) is the polynucleotide drawn from residues 32943 to 33644 of SEQ ID NO: 1, and SEQ ID NO: 56 represents the polypeptide deduced from SEQ ID NO: 57. ORF 29 (SEQ ID NO: 59) is the polynucleotide drawn from residues 34377 to 33637 of SEQ ID NO: 1, and SEQ ID NO: 58 represents the polypeptide deduced from SEQ ID NO: 59. ORF 30 (SEQ ID NO: 61) is the polynucleotide drawn from residues 34572 to 34907 of SEQ ID NO: 1, and SEQ ID NO: 60 represents the polypeptide deduced from SEQ ID NO: 61. ORF 31 (SEQ ID NO: 63) is the polynucleotide drawn from residues 34904 to 36583 of SEQ ID NO: 1, and SEQ ID NO: 62 represents the polypeptide deduced from SEQ ID NO: 63. ORF 32 (SEQ ID NO: 66) is the polynucleotide drawn from residues 23 to 1621 of SEQ ID NO: 64, and SEQ ID NO: 65 represents the polypeptide deduced from SEQ ID NO: 66. ORF 33 (SEQ ID NO: 68) is the polynucleotide drawn from residues 1702 to 2973 of SEQ ID NO: 64, and SEQ ID NO: 67 represents the polypeptide deduced from SEQ ID NO: 68. ORF 34 (SEQ ID NO: 70) is the polynucleotide drawn from residues 3248 to 4270 of SEQ ID NO: 64, and SEQ ID NO: 69 represents the polypeptide deduced from SEQ ID NO: 70. ORF 35 (SEQ ID NO: 72) is the polynucleotide drawn from residues 4452 to 5933 of SEQ ID NO: 64, and SEQ ID NO: 71 represents the polypeptide deduced from SEQ ID NO: 72. ORF 36 (SEQ ID NO: 75) is the polynucleotide drawn from residues 30 to 398 of SEQ ID NO: 73, and SEQ ID NO: 74 represents the polypeptide deduced from SEQ ID NO: 75. ORF 37 (SEQ ID NO: 77) is the polynucleotide drawn from residues 395 to 1372 of SEQ ID NO: 73, and SEQ ID NO: 76 represents the polypeptide deduced from

SEQ ID NO: 77. ORF 38 (SEQ ID NO: 79) is the polynucleotide drawn from residues 3388 to 1397 of SEQ ID NO: 73, and SEQ ID NO: 78 represents the polypeptide deduced from SEQ ID NO: 79. ORF 39 (SEQ ID NO: 81) is the polynucleotide drawn from residues 3565 to 5286 of SEQ ID NO: 73, and SEQ ID NO: 80 represents the polypeptide deduced from SEQ ID NO: 81. ORF 40 (SEQ ID NO: 83) is the polynucleotide drawn from residues 5283 to 7073 of SEQ ID NO: 73, and SEQ ID NO: 82 represents the polypeptide deduced from SEQ ID NO: 83. ORF 41 (SEQ ID NO: 85) is the polynucleotide drawn from residues 7108 to 8631 of SEQ ID NO: 73, and SEQ ID NO: 84 represents the polypeptide deduced from SEQ ID NO: 85. ORF 42 (SEQ ID NO: 87) is the polynucleotide drawn from residues 9371 to 8673 of SEQ ID NO: 73, and SEQ ID NO: 86 represents the polypeptide deduced from SEQ ID NO: 87. ORF 43 (SEQ ID NO: 89) is the polynucleotide drawn from residues 9762 to 9364 of SEQ ID NO: 73, and SEQ ID NO: 88 represents the polypeptide deduced from SEQ ID NO: 89.

Some open reading frames provided in the Sequence Listing, namely ORF 2 (SEQ ID NO: 5), ORF 5 (SEQ ID NO: 11), ORF 12 (SEQ ID NO: 25), ORF 13 (SEQ ID NO: 27), ORF 15 (SEQ ID NO: 31), ORF 17 (SEQ ID NO: 35), ORF 19 (SEQ ID NO: 39), ORF 20 (SEQ ID NO: 41), ORF 22 (SEQ ID NO: 45), ORF 24 (SEQ ID NO: 49), ORF 26 (SEQ ID NO: 53) and ORF 27 (SEQ ID NO: 55) initiate with non-standard initiation codons (eg. GTG – Valine, or CTG – Leucine) rather than standard initiation codon ATG methionine. All ORFs are listed with the appropriate M, V or L amino acids at the amino-terminal position to indicate the specificity of the first codon of the ORF. It is expected, however, that in all cases the biosynthesized protein will contain a methionine residue, and more specifically a formylmethionine residue, at the amino terminal position, in keeping with the widely accepted principle that protein synthesis in bacteria initiate with methionine (formylmethionine) even when the encoding gene specifies a non-standard initiation codon (e.g. Stryer BioChemistry 3rd edition, 1998, W.H. Freeman and Co., New York, pp. 752-754).

ORF 32 (SEQ ID NO: 65) is incomplete and contains a truncation of 10 to 20

amino acids from its carboxy terminus. This is due to incomplete sequence information between Contigs 2 and 3 (SEQ ID NOS: 64 and 73, respectively).

Deposits of E. coli DH10B vectors, each harbouring a cosmid clone (designated in FIGURE 12 as 046KM and 046KQ respectively) of a partial biosynthetic locus for the compound of Formula II from *Micromonospora sp.* strain 046-ECO11 and together spanning the full biosynthetic locus for production of the compound of Formula II have been deposited with the International Depositary Authority of Canada, Bureau of Microbiology, Health Canada, 1015 Arlington Street, Winnipeg, Manitoba, Canada R3E 3R2 on February 25, 2003. The cosmid clone designated 046KM was assigned deposit accession numbers IDAC 250203-06, and the cosmid clone designated 046KQ was assigned deposit accession numbers IDAC 250203-07. Cosmid 046KM covers residue 1 to residue 32,250 of Contig 1 (SEQ ID NO: 1). Cosmid 046KQ covers residue 21,700 of Contig 1 (SEQ ID NO: 1) to residue 9,762 of Contig 3 (SEQ ID NO: 73). The sequence of the polynucleotides comprised in the deposited strains, as well as the amino acid sequence of any polypeptide encoded thereby are controlling in the event of any conflict with any description of sequences herein.

The deposit of the deposited strains has been made under the terms of the Budapest Treaty on the International Recognition of the Deposit of Micro-organisms for Purposes of Patent Procedure. The deposited strains will be irrevocably and without restriction or condition released to the public upon the issuance of a patent. The deposited strains are provided merely as convenience to those skilled in the art and are not an admission that a deposit is required for enablement, such as that required under 35 U.S.C. §112. A license may be required to make, use or sell the deposited strains, and compounds derived therefrom, and no such license is hereby granted.

In order to identify the function of the proteins coded by the genes forming the biosynthetic locus for the production of the compound of Formula II the gene products of ORFs 1 to 43, namely SEQ ID NOS: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 65, 67, 69, 71, 74, 76,

78, 80, 82, 84, 86 and 88 were compared, using the BLASTP version 2.2.10 algorithm with the default parameters, to sequences in the National Center for Biotechnology Information (NCBI) nonredundant protein database and the DECIPHER® database of microbial genes, pathways and natural products (Ecopia BioSciences Inc. St.-Laurent, QC, Canada).

The accession numbers of the top GenBank™ hits of this BLAST analysis are presented in Table 14 along with the corresponding E values. The E value relates the expected number of chance alignments with an alignment score at least equal to the observed alignment score. An E value of 0.00 indicates a perfect homolog. The E values are calculated as described in Altschul *et al. J. Mol. Biol.*, 215, 403-410 (1990). The E value assists in the determination of whether two sequences display sufficient similarity to justify an inference of homology.

Table 14

ORF	Family	# aa	GenBank homology	Probability	% Identity	% Similarity	Proposed function of GenBank match
1	ABCC	571	NP_736627.1, 590aa	1.00E-107	222/496 (44.76%)	278/496 (56.05%)	ABC transporter Corynebacterium efficiens
			NP_600638.1, 510aa	5.00E-80	184/500 (36.8%)	260/500 (52%)	ABC transporter Corynebacterium efficiens
			NP_600638.1, 510aa	3.00E-12	58/195 (29.74%)	84/195 (43.08%)	ABC transporter Corynebacterium efficiens
2	RECH	689	CAC93719.1, 923aa	3.00E-17	57/158 (36.08%)	87/158 (55.06%)	regulator[Lechevalieria aerocolonigenes]
			BAC55205.1, 943aa	3.00E-12	51/170 (30%)	81/170 (47.65%)	transcriptional activator [Streptomyces sp.
			NP_631154.1, 932aa	3.00E-07	29/63 (46.03%)	40/63 (63.49%)	regulator. [Streptomyces coelicolor A3(2)
3	REGD	895	CAC93719.1, 923aa	3.00E-20	92/330 (27.88%)	142/330 (43.03%)	regulator [Lechevalieria aerocolonigenes]
			BAC55205.1, 943aa	1.00E-15	80/277 (28.88%)	101/277 (36.46%)	activator [Streptomyces sp. TP-A0274]
			NP_733725.1, 908aa	3.00E-12	95/339 (28.02%)	140/339 (41.3%)	regulator [Streptomyces coelicolor A3(2)]
4	IDSA	362	NP_601376.2, 371aa	2.00E-80	158/321 (49.22%)	208/321 (64.8%)	GGPP synthase [Corynebacterium glutamicum
			NP_738677.1, 366aa	3.00E-79	158/330 (47.88%)	204/330 (61.82%)	polyprenyl synthase,Corynebacterium efficiens
			NP_216689.1, 352aa	2.00E-78	153/331 (46.22%)	203/331 (61.33%)	idsA2 [Mycobacterium tuberculosis H37Rv]
5	MVKA	354	BAB07790.1, 345aa	2.00E-71	150/326 (46.01%)	193/326 (59.2%)	mevalonate kinase [Streptomyces sp. CL190]
			BAB07817.1, 334aa	5.00E-66	145/324 (44.75%)	185/324 (57.1%)	mevalonate kinase [Kitasatospora griseola]
			NP_720650.1, 332aa	3.00E-36	95/327 (29.05%)	157/327 (48.01%)	mevalonate kinase [Streptococcus mutans
6	DMDA	346	BAB07791.1, 350aa	2.00E-88	177/305 (58.03%)	199/305 (65.25%)	diphosphomevalonate decarboxylase [Streptomyces sp.
			BAB07818.1, 300aa	2.00E-69	145/275 (52.73%)	168/275 (61.09%)	mevalonate diPH decaroboxylase [Kitasatospora griseola]
			NP_785307.1, 325aa	3.00E-44	105/307 (34.2%)	141/307 (45.93%)	diphosphomevalonate decarboxylase [Lactobacillus plantarum
7	MVKP	369	BAB07792.1, 374aa	4.00E-93	183/365 (50.14%)	220/365 (60.27%)	phosphomevalonate kinase [Streptomyces sp. CL190]
			BAB07819.1, 360aa	6.00E-77	171/358 (47.77%)	202/358 (56.42%)	phosphomevalonate kinase [Kitasatospora griseola]
			AAG02442.1, 368aa	2.00E-31	102/354 (28.81%)	149/354 (42.09%)	3 phosphomevalonate kinase [Enterococcus faecalis]
8	IPPI	360	Q9KWF6, 364aa	1.00E-128	238/361 (65.93%)	269/361 (74.52%)	Isopentenyl-diphosphate delta-isomerase
			Q9KWG2, 363aa	1.00E-128	230/349 (65.9%)	270/349 (77.36%)	Isopentenyl-diphosphate delta-isomerase
			NP_814639.1, 347aa	5.00E-73	154/348 (44.25%)	212/348 (60.92%)	isopentenyl diphosphate isomerase [Enterococcus faecalis
9	HMGA	351	BAA70975.1, 353aa	1.00E-165	284/348 (81.61%)	317/348 (91.09%)	3-hydroxy-3-methylglutaryl coenzyme A reductase [Streptomyces sp.]
			BAA74565.1, 353aa	1.00E-160	282/347 (81.27%)	310/347 (89.34%)	3-hydroxy-3-methylglutaryl coenzyme A reductase [Kitasatospora

							griseola]
			BAA74566.1, 353aa	1.00E-155	277/347 (79.83%)	299/347 (86.17%)	3-hydroxy-3-methylglutaryl coenzyme A reductase [Streptomyces sp.]
10	KASH	391	BAB07795.1, 389aa	1.00E-148	260/386 (67.36%)	300/386 (77.72%)	3-hydroxy-3-methylglutaryl CoA synthase [Streptomyces sp. CL190]
			BAB07822.1, 346aa	1.00E-136	239/343 (69.68%)	268/343 (78.13%)	HMG-CoA synthase [Kitasatospora griseola]
			CAD24420.1, 388aa	6.00E-79	166/385 (43.12%)	210/385 (54.55%)	HMG-CoA synthase [Paracoccus zeaxanthinifaciens]
11	IPTN	290	NP_631248.1, 295aa	5.00E-22	79/282 (28.01%)	124/282 (43.97%)	hypothetical protein [Streptomyces coelicolor A3(2)]
			AAN65239.1, 324aa	5.00E-06	70/278 (25.18%)	112/278 (40.29%)	cloQ [Streptomyces roseochromogenes subsp. oscitans]
12	SPKG	370	AAM78435.1, 344aa	5.00E-48	112/208 (53.85%)	131/208 (62.98%)	two-component sensor [Streptomyces coelicolor A3(2)]
			NP_630507.1, 382aa	5.00E-48	112/208 (53.85%)	131/208 (62.98%)	sensor kinase [Streptomyces coelicolor A3(2)]
			ZP_00058991.1, 407aa	9.00E-34	88/198 (44.44%)	114/198 (57.58%)	Signal transduction histidine kinase [Thermobifida fusca]
13	RREB	220	NP_630508.1, 224aa	3.00E-79	148/220 (67.27%)	179/220 (81.36%)	regulatory protein [Streptomyces coelicolor A3(2)]
			ZP_00058992.1, 221aa	4.00E-67	129/218 (59.17%)	163/218 (74.77%)	Response regulator [Thermobifida fusca]
			NP_625364.1, 221aa	6.00E-66	134/222 (60.36%)	164/222 (73.87%)	response regulator [Streptomyces coelicolor A3(2)]
14	UNES	131	No hit	-	-	-	-
15	UNEZ	154	NP_649459.2, 628aa	7.60E-02	21/55 (38.18%)	33/55 (60%)	CG1090-PB [Drosophila melanogaster]
			NP_730819.1, 473aa	7.60E-02	21/55 (38.18%)	33/55 (60%)	CG1090-PA [Drosophila melanogaster]
			AAM11079.1, 428aa	7.60E-02	21/55 (38.18%)	33/55 (60%)	GH23040p [Drosophila melanogaster]
16	OXDS	661	NP_242948.1, 500aa	1.00E-52	129/433 (29.79%)	197/433 (45.5%)	unknown conserved protein [Bacillus halodurans]
			ZP_00091617.1, 480aa	3.00E-32	123/426 (28.87%)	175/426 (41.08%)	Putative multicopper oxidases [Azotobacter vinelandii]
			NP_252457.1, 463aa	1.00E-31	115/408 (28.19%)	170/408 (41.67%)	metallo-oxidoreductase [Pseudomonas aeruginosa PA01]
17	UNFD	129	NP_437360.1, 127aa	7.00E-33	73/121 (60.33%)	87/121 (71.9%)	bleomycin resistance protein family [Sinorhizobium meliloti]
			AAO91879.1, 123aa	1.00E-31	68/117 (58.12%)	86/117 (73.5%)	unknown [uncultured bacterium]
			NP_103287.1, 131aa	1.00E-23	59/122 (48.36%)	76/122 (62.3%)	unknown protein [Mesorhizobium loti]
18	UNFA	178					
19	CSMB	661	ZP_00137697.1, 769aa	1.00E-166	319/622 (51.29%)	408/622 (65.59%)	Anthranilate/para-aminobenzoate synthase [Pseudomonas aeruginosa
			NP_250594.1, 627aa	1.00E-166	319/622 (51.29%)	408/622 (65.59%)	phenazine biosynthesis protein PhzE [Pseudomonas aeruginosa PA01]
			ZP_00137701.1, 687aa	1.00E-166	319/622 (51.29%)	408/622 (65.59%)	Anthranilate/para-aminobenzoate synthas [Pseudomonas aeruginosa

20	AAKD	427	P41403, 421aa	1.00E-64	161/420 (38.33%)	214/420 (50.95%)	Aspartokinase (Aspartate kinase)
			ZP_00057166.1, 445aa	2.00E-64	154/415 (37.11%)	218/415 (52.53%)	Aspartokinases [Thermobifida fusca]
			AAD49567.1, 421aa	6.00E-64	152/412 (36.89%)	216/412 (52.43%)	aspartokinase subunit A [Amycolatopsis mediterranei]
21	ALDB	274	NP_275722.1, 266aa	2.00E-53	104/231 (45.02%)	147/231 (63.64%)	conserved protein [Methanothermobacter thermautotrophicus]
			NP_614692.1, 270aa	2.00E-52	104/240 (43.33%)	146/240 (60.83%)	Fructose-1,6-bisphosphate aldolase [Methanopyrus kandleri AV19]
			NP_615406.1, 267aa	2.00E-50	99/231 (42.86%)	141/231 (61.04%)	fructose-bisphosphate aldolase [Methanosarcina acetivorans str. C2A]
22	UNFC	367	NP_275723.1, 378aa	4.00E-46	116/308 (37.66%)	171/308 (55.52%)	conserved protein [Methanothermobacter thermautotrophicus]
			NP_614691.1, 402aa	2.00E-45	115/295 (38.98%)	163/295 (55.25%)	alternative 3-dehydroquinate synthase [Methanopyrus kandleri
			NP_248244.1, 361aa	2.00E-43	103/255 (40.39%)	150/255 (58.82%)	conserved hypothetical protein [Methanococcus jannaschii
23	HYDK	253	NP_577771.1, 247aa	4.00E-14	55/178 (30.9%)	87/178 (48.88%)	metal-dependent hydrolase [Pyrococcus furiosus DSM 3638]
			NP_142108.1, 247aa	1.00E-12	50/151 (33.11%)	78/151 (51.66%)	hypothetical protein PH0093 [Pyrococcus horikoshii]
			NP_125791.1, 248aa	1.00E-11	42/151 (27.81%)	76/151 (50.33%)	hypothetical protein [Pyrococcus abyssi]
24	ADSA	438	NP_070499.1, 433aa	2.00E-41	122/347 (35.16%)	171/347 (49.28%)	coenzyme F390 synthetase [Archaeoglobus fulgidus
			NP_618724.1, 434aa	5.00E-41	119/345 (34.49%)	171/345 (49.57%)	coenzyme F390 synthetase [Methanosarcina acetivorans
			NP_632700.1, 437aa	7.00E-41	121/345 (35.07%)	171/345 (49.57%)	Coenzyme F390 synthetase [Methanosarcina mazei Goe1]
25	HOXV	396	ZP_00027430.1, 442aa	8.00E-76	152/358 (42.46%)	211/358 (58.94%)	2-polyprenyl-6-methoxyphenol hydroxylase [Burkholderia fungorum]
			NP_627457.1, 420aa	1.00E-71	161/420 (38.33%)	216/420 (51.43%)	salicylate hydroxylase [Streptomyces coelicolor A3(2)]
			ZP_00033877.1, 403aa	2.00E-68	146/395 (36.96%)	200/395 (50.63%)	2-polyprenyl-6-methoxyphenol hydroxylase [Burkholderia fungorum]
26	SDRA	261	NP_391080.1, 261aa	6.00E-58	119/261 (45.59%)	149/261 (57.09%)	2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase [Bacillus subtilis]
			ZP_00059512.1, 260aa	1.00E-55	116/259 (44.79%)	144/259 (55.6%)	Dehydrogenase [Thermobifida fusca]
			AAG31126.1, 257aa	9.00E-55	117/257 (45.53%)	144/257 (56.03%)	MxcC [Stigmatella aurantiaca]
27	DHBS	224	Q51790, 207aa	7.00E-60	110/198 (55.56%)	142/198 (71.72%)	isochorismatase
			Q51518, 207aa	1.00E-58	110/198 (55.56%)	140/198 (70.71%)	isochorismatase
			NP_391077.1, 312aa	2.00E-58	106/203 (52.22%)	139/203 (68.47%)	isochorismatase [Bacillus subtilis]
28	SDRA	233	NP_103491.1, 242aa	9.00E-21	74/230 (32.17%)	112/230 (48.7%)	acyl-carrier protein reductase [Mesorhizobium loti]
			AAL14912.1, 245aa	1.00E-15	65/229 (28.38%)	100/229 (43.67%)	short-chain dehydrogenase [Rhizobium leguminosarum bv. trifolii]
			NP_902480.1, 235aa	7.00E-15	67/229 (29.26%)	100/229 (43.67%)	oxidoreductase [Chromobacterium violaceum
29	UNIQ	246	S18541, 281aa	4.50E-02	43/146 (29.45%)	63/146 (43.15%)	hypothetical protein 3 - Streptomyces coelicolor

			NP_629228.1, 281aa	5.90E-02	43/146 (29.45%)	63/146 (43.15%)	hypothetical protein [Streptomyces coelicolor A3(2)]
30	UNFE	111	ZP_00058149.1, 130aa	1.00E-10	35/97 (36.08%)	47/97 (48.45%)	membrane protein [Thermobifida fusca]
			NP_737701.1, 120aa	1.00E-09	37/111 (33.33%)	51/111 (45.95%)	hypothetical protein [Corynebacterium efficiens
			NP_827629.1, 118aa	7.00E-09	35/105 (33.33%)	51/105 (48.57%)	hypothetical protein [Streptomyces avermitilis MA-4680]
31	EFFT	559	ZP_00058148.1, 537aa	2.00E-67	165/517 (31.91%)	253/517 (48.94%)	Predicted symporter [Thermobifida fusca]
			NP_626090.1, 544aa	4.00E-66	162/521 (31.09%)	257/521 (49.33%)	transport protein [Streptomyces coelicolor A3(2)]
			NP_827630.1, 549aa	7.00E-63	160/523 (30.59%)	256/523 (48.95%)	sodium-dependent symporter [Streptomyces avermitilis
32	HOYH	532	AAM96655.1, 544aa	2.00E-92	206/526(39.16%)	279/526 (53.04%)	2,4-dihydroxybenzoate monooxygenase [Sphingobium chlorophenolicum]
			ZP_00029353.1, 543aa	1.00E-73	188/539 (34.88%)	263/539 (48.79%)	2-polyprenyl-6-methoxyphenol hydroxylase [Burkholderia fungorum]
			NP_769326.1, 569aa	5e-62	173/519 (33.33%)	251/519 (48.36%)	blr2686 [Bradyrhizobium japonicum] dbj
33	DAHP	423	T03226, 391aa	1.00E-111	207/383 (54.05%)	259/383 (67.62%)	hypothetical protein - Streptomyces hygroscopicus
			ZP_00137693.1, 405aa	3.00E-87	172/385 (44.68%)	233/385 (60.52%)	DAHP synthase [Pseudomonas aeruginosa UCBPP-PA14]
			NP_250592.1, 405aa	1.00E-86	169/380 (44.47%)	232/380 (61.05%)	phenazine biosynthesis protein PhzC [Pseudomonas aeruginosa
34	REGG	340	BAC53615.1, 346aa	1.00E-67	142/307 (46.25%)	192/307 (62.54%)	regulator protein [Streptomyces kasugaensis]
			S44506, 424aa	3.00E-66	141/305 (46.23%)	182/305 (59.67%)	regulator protein - Streptomyces glaucescens
			AAK81822.1, 348aa	1.00E-65	141/323 (43.65%)	192/323 (59.44%)	transcriptional regulator [Streptomyces lavendulae]
35	UNFJ	493	ZP_00073237.1, 678aa	7.00E-35	124/454 (27.31%)	197/454 (43.39%)	RTX toxins [Trichodesmium erythraeum IMS101]
			NP_484716.1, 433aa	3.00E-05	109/470 (23.19%)	172/470 (36.6%)	similar to vanadium chloroperoxidase [Nostoc sp.
			ZP_00067005.1, 667aa	7.40E-02	37/139 (26.62%)	52/139 (37.41%)	hypothetical protein [Microbulbifer degradans 2-40]
36	RECI	112	NP_627088.1, 125aa	3.00E-17	48/100 (48%)	59/100 (59%)	hypothetical protein . [Streptomyces coelicolor A3(2)]
			NP_846017.1, 109aa	7.00E-15	40/101 (39.6%)	60/101 (59.41%)	hypothetical protein [Bacillus anthracis str. Ames]
			NP_241272.1, 174aa	9.00E-15	39/106 (36.79%)	62/106 (58.49%)	unknown conserved protein [Bacillus halodurans]
37	UNIQ	325	NP_422203.1, 187aa	1.00E-03	24/61 (39.34%)	36/61 (59.02%)	hypothetical protein [Caulobacter crescentus CB15]
38	OXAH	663	ZP_00058724.1, 659aa	0.00E+00	370/647 (57.19%)	435/647 (67.23%)	Acyl-CoA dehydrogenases [Thermobifida fusca]
			AAB97825.1, 433aa	5.00E-93	203/446 (45.52%)	251/446 (56.28%)	acyl-CoA oxidase [Myxococcus xanthus]
			AAF14635.1, 694aa	5.00E-85	211/565 (37.35%)	292/565 (51.68%)	1 acyl-CoA oxidase [Petroselinum crispum]
39	ABCA	537	T14162, 574aa	9.00E-62	189/509 (37%)	240/509 (47%)	hABC transport protein - Mycobacterium smegmatis
			NP_624808.1	4.00E-60	184/540 (35%)	251/540 (46%)	ABC transporter [Streptomyces coelicolor A3(2)]

			NP_822745.1	8.00E-32	124/392 (31%)	168/392 (42%)	ABC transportert [Streptomyces avermitilis MA-4680]
40	ABCA	596	T14180, 1122aa	1.00E-107	236/594 (39.73%)	300/594 (50.51%)	exiT protein - Mycobacterium smegmatis
			AAC82548.1, 589aa	1.00E-107	234/583 (40.14%)	295/583 (50.6%)	unknown [Mycobacterium smegmatis]
			NP_624810.1, 601aa	3.00E-97	222/593 (37.44%)	283/593 (47.72%)	ABC-transporter [Streptomyces coelicolor A3(2)]
41	UNIQ	507	NP_831570.1, 676aa	8.00E-07	62/262 (23.66%)	116/262 (44.27%)	methyltransferases [Bacillus cereus
			NP_655735.1, 676aa	2.00E-06	61/262 (23.28%)	116/262 (44.27%)	ubiE/COQ5 methyltransferase family [Bacillus anthracis
			NP_844290.1, 681aa	2.00E-06	61/262 (23.28%)	116/262 (44.27%)	hypothetical protein [Bacillus anthracis str. Ames]
42		232	NP_830809.1, 208aa	8.00E-08	46/210 (21.9%)	74/210 (35.24%)	Transporter, LysE family [Bacillus cereus]
			NP_844737.1, 210aa	2.00E-07	46/210 (21.9%)	74/210 (35.24%)	homoserine/threonine efflux protein[Bacillus anthracis
			NP_655752.1, 208aa	1.00E-06	47/210 (22.38%)	75/210 (35.71%)	LysE, LysE type translocator [Bacillus anthracis
43		132	NP_827272.1, 127aa	4.00E-09	38/107 (35.51%)	52/107 (48.6%)	hypothetical protein [Streptomyces avermitilis MA-4680]
			NP_246491.1, 112aa	5.90E-02	21/94 (22.34%)	44/94 (46.81%)	unknown [Pasteurella multocida]

The ORFs encoding proteins involved in the biosynthesis of compounds of Formula II are assigned a putative function and grouped together in families based on sequence similarity to known proteins. To correlate structure and function, the protein families are given a four-letter designation used throughout the description and figures as indicated in Table 15. The meaning of the four letter designations is as follows: AAKD designates an amino acid kinase; ABCA and ABCC designate ABC transporters; ADSA designates an amide synthetase; ALDB designates an aldolase function; CSMB designates a chorismate transaminase; DAHP designates a 3,4-dideoxy-4-amino-D-arabino-heptulosonic acid 7-phosphate synthase activity; DHBS designates a 2,3-dihydro-2,3-dihydroxybenzoate synthase activity; DMDA designates a diphosphomevalonate decarboxylase; EFFT designates an efflux protein; HMGA designates a 3-hydroxy-3-methylglutaryl-CoA reductase; HOXV designates a monooxygenase activity; HOYH designates a hydroxylase/decarboxylase activity; HYDK designates a hydrolase activity; IDSA designates an isopentenyl diphosphate synthase; IPPI designates an isopentenyl diphosphate isomerase; IPTN designates an isoprenyltransferase; KASH designates 3-hydroxy-3-methylglutaryl-CoA synthase; MVKA designates a mevalonate kinase; MVPK designates a phosphomevalonate kinase; OXAH designates an acylCoA oxidase; OXDS designates an oxidoreductase; RECH, RECI, REGD, REGG and RREB designate regulators; SDRA designates a dehydrogenase/ketoreductase, SPKG designates a sensory protein kinase; UNES, UNEZ, UNFA, UNFC, UNFD, UNFE, UNFJ and UNIQ designate proteins of unknown function.

Table 15

FAMILY	FUNCTION
AAKD	amino acid kinase; strong homology to primary aspartate kinases, converting L-aspartate to 4-phospho-L-aspartate
ABCA	ABC transporter
ABCC	ABC transporter
ADSA	adenylating amide synthetase
ALDB	aldolase; similarity to fructose-1,6-biphosphate aldolase that generates D-glyceraldehyde-3Ph, precursor of D-erythrose-4Ph involved in the shikimate pathway

CSMB	chorismate transaminase, similarity to anthranilate synthase
DAHP	DAHP synthase, class II; involved in formation of aminoDAHP from PEP and erythrose-4-phosphate
DHBS	2,3-dihydro-2,3-dihydroxybenzoate synthase (isochorismatase)
DMDA	diphosphomevalonate decarboxylase (mevalonate pyrophosphate decarboxylase)
EFFT	efflux protein
HMGA	HMG-CoA reductase; converts 3-hydroxy-3-methylglutaryl-CoA to mevalonate plus CoA in isoprenoid biosynthesis
HOXV	FAD monooxygenase; shows homology to a variety of monooxygenases including salicylate hydroxylases, zeaxanthin epoxidases
HOYH	hydroxylase/decarboxylase; FAD-dependent monooxygenase
HYDK	hydrolase
IDSA	isoprenyl diphosphate synthase, catalyzes the addition of 2 molecules of isopentenyl pyrophosphate to dimethylallyl pyrophosphate to generate GGPP
IPPI	isopentenyl diphosphate isomerase, catalyzes the isomerization of IPP to produce dimethylallyl diphosphate
IPTN	isoprenyltransferase; catalyzes covalent N-terminal attachment of isoprenyl units to amide groups of nitrogen-containing heterocycle rings
KASH	HMG-CoA synthase; condenses acetyl-CoA with acetoacetyl-CoA to form 3-hydroxy-3-methylglutaryl-CoA
MVKA	mevalonate kinase; converts mevalonate to 5-phosphomevalonate in the mevalonate pathway of isoprenoid biosynthesis
MVKP	phosphomevalonate kinase; converts 5-phosphomevalonate to 5-diphosphomevalonate in the mevalonate pathway of isoprenoid biosynyhesis
OXAH	acyl CoA oxidase
OXDS	oxidoreductase
RECH	regulator
RECI	regulator; similarity to PadR transcriptional regulators involved in repression of phenolic acid metabolism
REGD	transcriptional regulator; relatively large regulators with an N-terminal ATP-binding domain containing Walker A and B motifs and a C-terminal LuxR type DNA-binding domain
REGG	regulator
RREB	response regulator; similar to response regulators that are known to bind DNA and act as transcriptional activators
SDRA	dehydrogenase/ketoreductase, NAD-dependent
SPKG	sensory protein kinase, two component system
UNES	unknown function
UNEZ	unknown function
UNFA	unknown function
UNFC	unknown function

UNFD	unknown function
UNFE	putative membrane protein
UNFJ	unknown function
UNIQ	unknown function

Biosynthesis of the compound of Formula II involves the action of various enzymes that synthesize the three building blocks of the compound, namely the farnesyl-diphosphate component (FIGURE 13), the 3-hydroxy-anthranilate-adenylate component (FIGURE 14a) and the 2-amino-6-hydroxy-benzoquinone component (FIGURE 14b) that are subsequently condensed to form the final compound (FIGURE 15).

The farnesyl-diphosphate biosynthesis involves the concerted action of seven enzymes (FIGURE 13). ORF 10 (KASH) (SEQ ID NO: 20) encodes a hydroxymethylglutaryl-CoA synthase that catalyzes an aldol addition of acetyl-CoA onto acetoacyl-CoA to yield 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). This product is subsequently reduced through the action of ORF 9 (HMGA) (SEQ ID NO: 18) to form mevalonic acid (MVA). ORF 5 (MVKA) (SEQ ID NO: 10) phosphorylates mevalonate to 5'-phosphomevalonate using ATP as the phosphate donor. The next step in the farnesyl-diphosphate biosynthesis is the phosphorylation reaction of the 5'-phosphomevalonate to 5'-pyrophosphomevalonate (DPMVA) that is catalyzed by ORF 7 (MVKP) (SEQ ID NO: 14). Subsequent decarboxylation of 5'-pyrophosphomevalonate catalyzed by ORF 6 (DMDA) (SEQ ID NO: 12) yields isopentenyl diphosphate (IPP) which is then converted to dimethylallyldiphosphate (DMADP) through the action of ORF 8 (IPPI) (SEQ ID NO: 16) that has isomerase enzymatic activity. The final step in the biosynthesis of farnesyl-diphosphate is the condensation of one molecule of dimethylallyldiphosphate with two molecules of isopentenyl diphosphate catalyzed by the isoprenyl diphosphate synthase ORF 4 (IDSA) (SEQ ID NO: 8). The described pathway involved in synthesis of farnesyl-diphosphate is entirely consistent with related mevalonate pathways described in other actinomycete species (Takagi *et al.*, *J. Bacteriol.* 182, 4153-4157, (2000)).

Biosynthesis of the 3-hydroxy-anthranilate component involves the use of

precursors derived from the shikimate pathway (FIGURE 14a). Chorismic acid is transaminated through the action of ORF 19 (CSMB) (SEQ ID NO: 38) to form aminodeoxyisochorismic acid. This enzyme resembles anthranilate synthases and is likely to catalyze specifically the transfer of the amino group using glutamine as the amino donor. The next step involves isochorismatase activity and is mediated by ORF 27 (DHBS) (SEQ ID NO: 54). This reaction consists in the removal of the pyruvate side chain from aminodeoxyisochorismic acid to form 6-amino-5-hydroxy-cyclohexa-1,3-dienecarboxylic acid. This compound is subsequently oxidized through the action of ORF 26 (SDRA) (SEQ ID NO: 52) yielding 3-hydroxy-anthranilic acid. ORF 24 (ADSA) (SEQ ID NO: 48) catalyzes the activation of 3-hydroxy-anthranilic acid through adenylation generating the 3-hydroxy-anthranilate-adenylate component (FIGURE 14a).

Biosynthesis of the 2-amino-6-hydroxy-benzoquinone component of the compound of Formula II, requires components derived from the aminoshikimate pathway. FIGURE 14b depicts the series of enzymatic reactions involved in the biosynthesis of this constituent. ORF 21 (ALDB) (SEQ ID NO: 42) resembles aldolases involved in the generation of precursors of D-erythrose-4-phosphate which is part of the aminoshikimate pathway used for the generation of 2-amino-6-hydroxy-[1,4]-benzoquinone. ORF 33 (DAHP) (SEQ ID NO: 67) catalyzes the initial step in the aminoshikimate pathway that corresponds to the formation of 3,4-dideoxy-4-amino-D-arabino-heptulosonic acid 7-phosphate (amino DAHP) from phosphoenolpyruvate (PEP) and erythrose 4-phosphate (E-4Ph). Subsequent reactions leading to 3-amino-5-hydroxy-benzoic acid are catalyzed by enzymes provided by primary metabolism biosynthetic pathways present in *Micromonospora sp.* strain 046-ECO11. ORF 25 (HOXV) (SEQ ID NO: 50) hydroxylates 3-amino-5-hydroxy-benzoic acid at position 2, generating 3-amino-2,5-dihydroxy-benzoic acid. This intermediate is further modified by ORF 32 (HOYH) (SEQ ID NO: 65) that catalyzes a decarboxylative oxidation reaction yielding 6-amino-benzene-1,2,4-triol. A final oxidation reaction is performed by ORF 16 (OXDS) (SEQ ID NO: 32) yielding 2-amino-6-hydroxy-[1,4]-benzoquinone (FIGURE 14b).

Assembly of the three components resulting in the compound of Formula II is

catalyzed by ORFs 24 and 11 (FIGURE 15). ORF 24 (ADSA) (SEQ ID NO: 48) catalyzes the condensation of the adenylated 3-hydroxy-anthranilate with the 2-amino-6-hydroxy-[1,4]-benzoquinone component. A spontaneous condensation between the free amino group of the 3-hydroxy-anthranilate and one of the carbonyl groups present on the 2-amino-6-hydroxy-[1,4]-benzoquinone component occurs yielding a dibenzodiazepinone intermediate. This compound is further modified through transfer of the farnesyl group of the farnesyl-diphosphate intermediate onto the nitrogen of the amide of the dibenzodiazepinone catalyzed by ORF 11 (IPTN) (SEQ ID NO: 22) and resulting in the formation of the compound of Formula II (FIGURE 15).

Additional ORFs, namely ORF 2 (RECH) (SEQ ID NO: 4), ORF 3 (REGD) (SEQ ID NO: 6), ORF 12 (SPKG) (SEQ ID NO: 24), ORF 13 (RREB) (SEQ ID NO: 26), ORF 34 (REGG) (SEQ ID NO: 69) and ORF 36 (RECI) (SEQ ID NO: 74) are involved in the regulation of the biosynthetic locus encoding the compound of Formula II. Other ORFs, namely ORF 1 (ABCC) (SEQ ID NO: 2), ORF 31 (EFFT) (SEQ ID NO: 62), ORFs 39 and 40 (ABCA) (SEQ ID NOS: 80 and 82, respectively) and ORF 42 (SEQ ID NO: 86) are involved in transport. Other ORFs involved in the biosynthesis of the compound of Formula II include ORF 20 (AAKD) (SEQ ID NO: 40), ORF 23 (HYDK) (SEQ ID NO: 46), ORF 38 (OXAH) (SEQ ID NO: 78) as well as ORFs 14, 15, 17, 18, 22, 29, 30, 35, 37, 41 and 43 (SEQ ID NOS: 28, 30, 34, 34, 44, 58, 60, 71, 76, 84 and 88, respectively) of unknown function.

TABLE 16: PREFERRED MEDIA COMPOSITION FOR PRODUCTION OF ECO-04601

Component	QB	MA	KH	RM	JA	FA
pH*[5]	7.2	7.5	7	6.85	7.3	7.0
Glucose	12		10	10		10
Sucrose				100		
Lactose						
Cane molasses						15
Corn starch					30	
Soluble starch	10	25				
Potato dextrin			20			40
Corn steep solid						
Corn steep liquor	5				15	
Dried yeast		2				
Yeast extract			5			
Malt extract					35	
Pharmamedia™	10				15	
Glycerol						
NZ-Amine			5			10
Soybean powder		15				
Soybean flour						
Meat extract						
Bacto-peptone						
$MgSO_4.7H_2O$						1
$MgCl_2.\ 6H_2O$						
$CaCO_3$		4	1		2	2
NaCl		5				
$(NH_4)_2\ SO_4$		2				
$K_2\ SO_4$				0.25		
$MnCl_2.4H_2O$						
$MgCl_2.6H_2O$				10		
$FeCl_2.4H_2O$						
$ZnCl_2$						
Na_2HPO_4						3
Thiamine						
Casamino acid				0.1		
Proflo oil	4					
MOPS				21		
Trace element solution *[3] ml/L				2		

Unless otherwise indicated all the ingredients are in gm/L.
*[3] Trace elements solution contains: $ZnCl_2$ 40 mg; Fe $Cl_3\ 6H_2O$ (200 mg); $CuCl_2\ 2H_2O$ (10 mg); $MnCl_2.4H_2O$; $Na_2B_4O_7.10H_2O$ (10mg); $(NH_4)_6\ MO_7O_{24}\ .4H_2O$ (10 mg) per litre.
*[5] The pH is to adjusted as marked prior to the addition of $CaCO_3$.

All patents, patent applications, and published references cited herein are hereby incorporated by reference in their entirety. While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the scope of the invention encompassed by the appended claims.